REGULATION OF THE ENDONUCLEASE ACTIVITY OF TYPE I RESTRICTION-MODIFICATION SYSTEMS

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A thesis presented for the degree of Doctor of Philosophy

Institute of Cell and Molecular Biology

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October, 1999

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Noreen Murray, for teaching me both science and English, for her encouragement, support and ideas.

Many thanks to my second supervisor, David Leach, for his useful suggestions on the project.

I would like to acknowledge all of the people from Noreen's and Dave's labs, most particularly Annette, Dave, Laurie, Lynn and Mary, for their advise and for teaching me new techniques. Thanks to people from other labs, including Andrei, Angela, Dima, Eve, John, Ivan, Laura, Ken, Kristina, Millie, Richard, Sean, Taciana, Willie and Zhenya, who helped me with strains, equipment, computers or other things.

I am very grateful to my Russian and Byelorussian friends for the opportunity to speak our native language far away from home.

I want to thank my family for their weekly letters, patience and love.

Most of all, I would like to acknowledge Noreen and Ken, founders of The Darwin Trust, not only for the Fellowship but also for their wonderful idea to give a chance to Eastern Europeans to enjoy doing modern science. This thesis is devoted to them and the abbreviation NK that was used to name strains and plasmids stands for *N*oreen and *K*en.

ABSTRACT

Efficient acquisition of the genes (*hsdR, M* and *S*) that specify *Eco*KI and *Eco*AI, representatives of two families of type I restriction and modification (R-M) systems, was shown to require a product of an unknown gene *hsdC*. The *hsdC* mutant is shown to have a mutation in *clpX*. ClpX and ClpP, the components of ClpXP protease, are necessary for the efficient transmission of the *hsd* genes by conjugation, transformation and P1 transduction. Inactivation of *clpX* leads to a bigger barrier than a similar mutation in *clpP* consistent with a chaperone activity of ClpX in the absence of ClpP. The establishment of the modification activity of *Eco*KI is not dependent on *clpX* and takes about 12 generations to reach its maximal activity in methylating incoming phage DNA. This lag probably reflects the time necessary to complete the methylation of bacterial chromosomes.

Modification, once established, has been assumed to provide adequate protection against a resident restriction system. However, unmodified targets may be generated in the DNA of an hsd^+ bacterium as the result of replication errors or recombination-dependent repair. The presence of unmodified target sequences for type I restriction-modification systems on bacterial chromosomes does not influence the survival of hsd^+ bacteria due to ClpXP-dependent regulation of the endonuclease activity. In such bacteria, HsdR, the polypeptide of the R-M complex essential for restriction but not modification, is degraded in the presence of ClpXP and therefore the bacteria show a temporary drop in restriction activity, referred to as restriction alleviation. The delayed detection of restriction activity followed by the establishment of a new specificity can be considered as a case of restriction alleviation.

The data obtained support a model in which the HsdR component of a type I restriction endonuclease becomes a substrate for proteolysis after the endonuclease has bound to unmodified target sequences on the chromosome, but before completion of the pathway that would result in DNA breakage. It remains unclear how the restriction-modification systems distinguish between unmethylated host and foreign DNA. The latter is degraded while the former is protected from cleavage by ClpXP-dependent proteolysis of HsdR.

ABBREVIATIONS

AdoMet	S-adenosyl-L-methionine
2-AP	2-aminopurine
ATP	adenosine triphosphate
bp	base pair(s)
c.f.u.	colony forming units
DSB	double-strand break(s)
dsDNA	double-stranded DNA
EDTA	ethylenediaminetetraacetic acid
e.o.p.	efficiency of plating
g	standard acceleration due to gravity
kb	kilobase(s)
kD	kilodalton(s)
log	logarithm(ic)
MMS	methyl methanesulfonate
OD	optical density
PEG	polyethylene glycol
p.f.u.	plaque forming units
PVDF	polyvinylidene difluoride
RA	restriction alleviation
R-M	restriction and modification
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
SSC	standard saline citrate
TEMED	N,N,N′,N′-tetramethylethylenediamine
ts	temperature sensitive
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
Δ	deletion

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CHAPTER 1. GENERAL INTRODUCTION

1.1. Restriction and modification systems.

The phenomenon of restriction was first observed in the early 1950s (Luria & Human, 1952; Bertani & Weigle, 1953). Some bacterial viruses grown on one strain could infect this strain much more efficiently than some other strains. Once they have propagated on a new host the phage progeny acquired the ability to infect this new strain effectively but their growth on the original strain became inhibited, or restricted (Bertani & Weigle, 1953; Arber & Dussoix, 1962). It seemed as if the phage had an "imprint" of the bacteria in which they were propagated and the "imprint" allowed the phage to infect this particular strain effectively. If the phage lacked the appropriate "imprint" then their propagation was restricted by a new host. The "imprint" was found to be carried by phage DNA (Dussoix & Arber, 1962) and later experiments showed that it was methylation (Brockes *et al.*, 1972; Kuehnlein & Arber, 1972; Smith *et al.*, 1972). If DNA is not methylated it becomes a substrate for endonucleolytic cleavage by a restriction enzyme (Linn & Arber, 1968; Meselson & Yuan, 1968). Since then, many restriction and modification systems have been described. They are different in their structure, mode of action and regulation and have been divided in three different types. In addition, there are restriction systems specific for modified DNA (see below) that cannot be covered by this classification.

1.1.1. Type I R-M systems.

Type I R-M systems are found in a variety of bacteria such as *E.coli* (Bertani & Weigle, 1953; Meselson & Yuan, 1968), *Salmonella* species (Bullas & Colson, 1975), *Citrobacter freundii* (Daniel *et al.*, 1988), *Mycoplasma pulmonis* (Dybvig & Yu, 1994), *Bacillus subtilis* (Xu *et al.*, 1995), *Pasteurella haemolytica* (Highlander & Garza, 1996), *Klebsiella pneumoniae* (Lee *et al.*, 1997) and others. It seems that they are rather commonly found in bacterial species. Analysis of the sequenced genomes of 15 bacterial species led to the identification of 16 type I R-M systems belonging to

11 of the 15 bacterial species (Roberts, 1998) most of which had not been known to contain type I R-M systems.

Type I R-M systems are encoded by *hsd* genes (for *h*ost *s*pecificity of *D*NA) located either in the bacterial chromosome or in a plasmid. Those in the chromosome are in the region equivalent to 98.5′ of the *E.coli* K-12 chromosome, closely linked with *serB* (Boyer, 1964; Glover & Colson, 1969; Bullas & Colson, 1975) and can be considered as multiple alleles (Daniel *et al.*, 1988). The *hsd* locus comprises three genes (Glover, 1970), *hsdR* (for *r*estriction), *hsdM* (for *m*ethylation), and *hsdS* (for *s*pecificity). *hsdM* and *hsdS* are transcribed from a promoter upstream of *hsdM*, and *hsdR* has its own promoter. Transcription occurs in the same direction for all the three genes (Sain & Murray, 1980).

HsdR, HsdM and HsdS polypeptides are structural components of multisubunit complexes (Eskin & Linn, 1972; Lautenberger & Linn, 1972; Meselson *et al.*, 1972), they do not possess any enzymatic activity on their own. HsdM and HsdS form M_2S_1, a complex that shows only methylation activity (Lautenberger & Linn, 1972; Suri *et al.*, 1984; Taylor *et al.*, 1992; Dryden *et al.*, 1993). The restriction enzyme requires HsdR in addition to HsdM and HsdS to form the $R_2M_2S_1$ complex (Eskin & Linn, 1972; Meselson *et al.*, 1972; Weiserova *et al.*, 1993; Dryden *et al.*, 1997) that retains the methylation activity (Haberman *et al.*, 1972; Vovis *et al.*, 1974) but also possesses endonuclease activity (Eskin & Linn, 1972; Haberman *et al.*, 1972; Vovis *et al.*, 1974; Suri *et al.*, 1984).

Type I systems are subdivided into families on the basis of complementation tests (Arber & Linn, 1969; Boyer & Roulland-Dussoix, 1969; Glover, 1970; Fuller-Pace *et al.*, 1985; Price *et al.*, 1987), DNA-cross hybridisation (Sain & Murray, 1980; Daniel *et al.*, 1988; Barcus *et al.*, 1995) and immunological cross reactivity (Murray *et al.*, 1982; Price *et al.*, 1987). For any two systems within a family, DNA sequences coding for HsdR, HsdM and the conservative domains of HsdS are so similar that the DNA can be cross hybridised. At the protein level this similarity results in the ability of homologous subunits to substitute for each other in the formation of functional complexes and to react with antiserum raised against another enzyme from the same family. Each of these three tests fails for two representatives from different families.

Four families (IA, IB, IC and ID) have been identified (Bickle & Kruger, 1993; Barcus *et al.*, 1995; Titheradge *et al.*, 1996).

Systems from the same family differ only in non-conserved regions of their S subunits, named target recognition domains, which define DNA target sequences recognised by different R-M systems (Fuller-Pace *et al.*, 1984; Nagaraja *et al.*, 1985b; Gann *et al.*, 1987). S subunits are involved in the recognition of target sites by both methylation and restriction enzymes (Cowan *et al.*, 1989; Chen *et al.*, 1995; O'Neill *et al.*, 1998). Conserved regions of HsdS are thought to be involved in subunit-subunit interactions.

Both M_2S_1 and $R_2M_2S_1$ complexes bind a cofactor S-adenosylmethionine (AdoMet) which is used as a donor of methyl groups to methylate adenine residues in DNA target sequences (Haberman *et al.*, 1972). Target sequences recognised by type I enzymes are asymmetric and consist of two recognition components, one of which is generally a 3 bp sequence and the other either 4 or 5 bp, separated by a non-specific spacer of 6 to 8 bp (Lautenberger *et al.*, 1978; Ravetch *et al.*, 1978; Kan *et al.*, 1979; Kroger & Hobom, 1984; Suri *et al.*, 1984; Nagaraja *et al.*, 1985a, 1985b; Price *et al.*, 1987). The two target recognition domains of HsdS each recognises one half of the bipartite target (Fuller-Pace *et al.*, 1984; Nagaraja *et al.*, 1985c; Gann *et al.*, 1987). The two adenine residues to be methylated are located in different recognition components and in the opposite DNA strands. DNA replication separates methylated strands and produces hemimethylated DNA which is a substrate for methylation but not for restriction (Vovis *et al.*, 1974). Type IA and IC enzymes methylate hemimethylated DNA much better than unmethylated (Vovis *et al.*, 1974; Suri *et al.*, 1984; Dryden *et al.*, 1993; Taylor *et al.*, 1993), while *Eco*AI (a type IB system) shows no preference (Fuller-Pace *et al.*, 1985; Suri & Bickle, 1985). It seems likely that poor methylation of unmethylated DNA makes it more difficult for incoming DNA to escape from restriction.

Restriction occurs only on unmethylated DNA (Vovis *et al.*, 1974). When a restriction enzyme $R_2M_2S_1$ binds an unmodified target sequence the restriction pathway is activated and results in DNA cleavage by the nuclease. This reaction requires AdoMet, ATP and Mg^{2+} (Linn & Arber, 1968; Meselson & Yuan, 1968; Yuan & Meselson, 1970; Boyer *et al.*, 1971). *Eco*KI does not require either AdoMet

or ATP to bind DNA nonspecifically (Powell *et al.*, 1998). The cofactors assist the enzyme to recognise its target site. Exonuclease III footprinting experiments show that *Eco*KI, bound to its unmethylated or hemimethylated DNA target sequence in the absence of ATP and AdoMet, protects 42-46 bp of DNA from digestion by the exonuclease (Powell *et al.*, 1998). In the presence of the cofactors the enzyme undergoes a conformational change which results in the protection of a shorter sequence of 30 bp. The enzyme has reduced affinity for modified DNA; DNA with a fully methylated target is not protected by *Eco*KI from the exonuclease III-dependent degradation.

DNA cleavage by type I enzymes occurs up to several kb away from their recognition sites (Horiuchi & Zinder, 1972). The enzymes bound to target sequences reach cleavage sites via ATP-dependent DNA translocation (Yuan *et al.*, 1980). Based on the products of *in vitro* restriction of unmodified DNA of the phage T7 by *Eco*KI, Studier & Bandyopadhyay (1988) suggested a model according to which the enzymes, bound to unmethylated DNA targets, initiate ATP-dependent DNA translocation in both directions. While the protein complex stays bound to the target, the HsdR subunits pull the DNA past themselves. When two complexes, active on the same DNA molecule, collide a DSB is made and therefore cleavage sites for type I enzymes are between their recognition sequences (Fig. 1.1).

The idea of translocation is supported by predictions of structural similarity between helicases and HsdR. RNA and DNA helicases have seven conserved sequences, DEAD-box motifs, involved in the NTP-dependent duplex unwinding (Gorbalenya *et al.*, 1988). The DEAD-box motifs found in HsdR (Gorbalenya & Koonin, 1991; Webb *et al.*, 1996; Davies *et al.*, 1998) were shown to be associated with ATP-dependent DNA translocation activity (Davies *et al.*, 1999b). A single amino acid substitution in any of them abolishes ATPase activity and consequently blocks restriction (Webb *et al.*, 1996; Davies *et al.*, 1998). Mutant HsdR subunits still can form $R_2M_2S_1$ complexes and the latter bind DNA in a site-specific manner and undergo the ATP-dependent conformational change but DNA cleavage does not occur (Davies *et al.*, 1998). Therefore translocation seems to be a prerequisite for the endonuclease reaction.

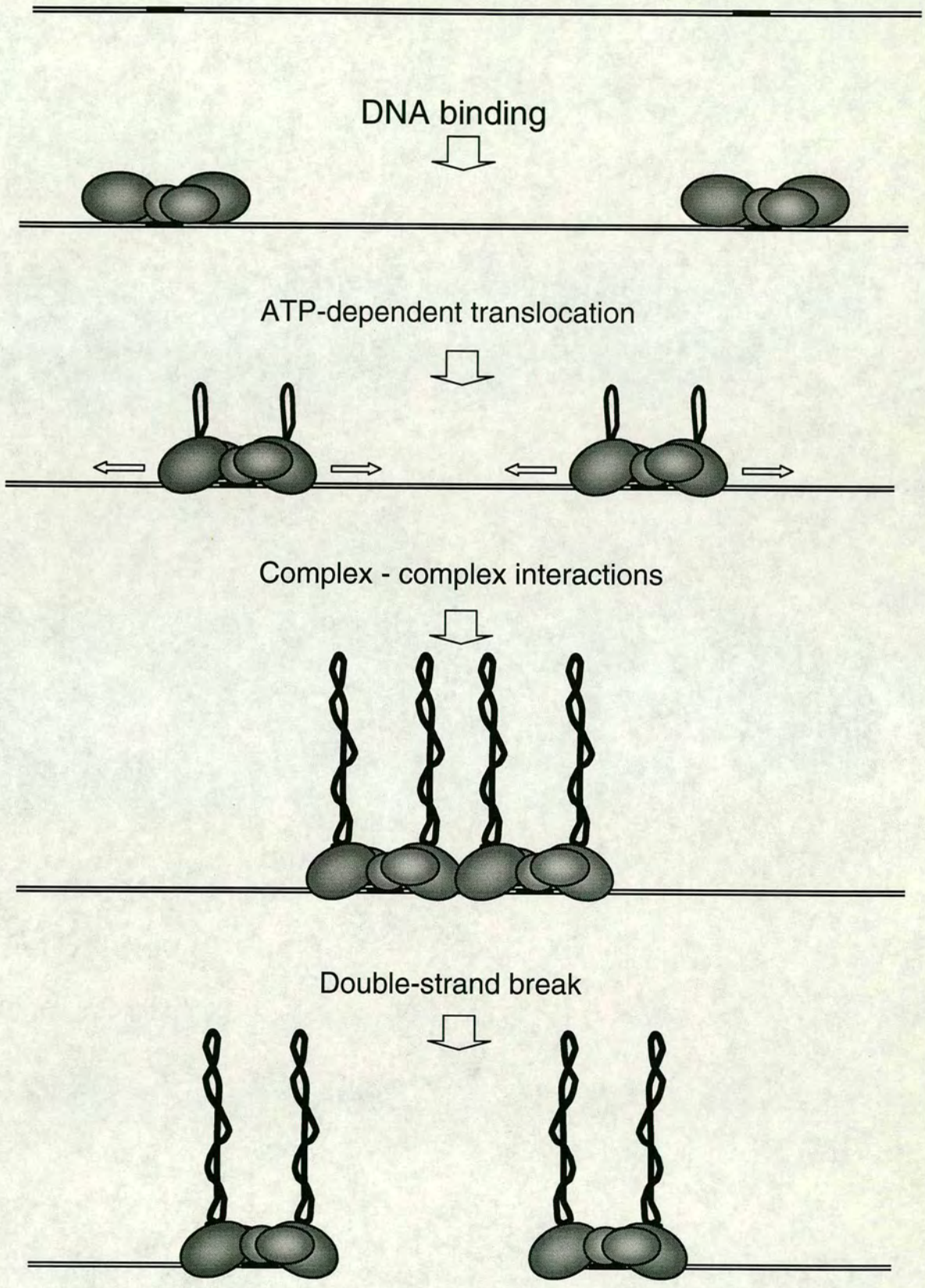


Fig. 1.1. Molecular mechanism of restriction by type I endonucleases.

Endonucleolytic cleavage of DNA occurs when translocation cannot proceed any further because of an obstacle (Janscak *et al.*, 1999a). When two complexes translocating the same molecule of DNA towards themselves collide they become such obstacles for each other and a DSB is produced at the site of the collision. It has been shown that each complex is able to cleave both DNA strands (Janscak *et al.*, 1999a). There is no specific interaction between complexes required for the cleavage reaction since a Holliday junction, which blocks translocation, also can stimulate endonuclease activity of a type I enzyme (Janscak *et al.*, 1999a).

Recent experiments indicate that the region X (Titheradge *et al.*, 1996), a conservative sequence in the N-terminal part of HsdR of different type I systems and common for different nucleases, is involved in DNA cleavage. Mutations in the region X lead to the loss of restriction proficiency but the enzymes still retain their ability to translocate DNA (Davies *et al.*, 1999b; Janscak *et al.*, 1999b). The predicted secondary structure of the region including motif X resembles that of the active site of other endonucleases (Davies *et al.*, 1999a) in which binding of the divalent cation, Mg^{2+}, is responsible for the cleavage of a single strand of DNA.

Type I systems are the most complex of all R-M systems. It is not clear how many biological advantages derive from this complexity. One may be the evolution of new specificities. The structure of HsdS, where two TRDs are separated by a central conservative region and each TRD recognises a half of the target sequence independently of each other, leaves an option to create an enzyme with a new specificity via homologous recombination between two *hsdS* genes from the same family. DNA coding for the conservative region can provide homology for recombination that would lead to reassortment of the TRDs. The resultant HsdS would recognise the 5′half of its target like one of the parent enzymes and the 3′half like the other. Such recombination leading to a new specificity was observed *in vivo* as the result of P1-mediated transduction between two strains with type IA specificities (Bullas *et al.*, 1976; Fuller-Pace *et al.*, 1984). Similarly, an enzyme with a novel specificity can be obtained for the IB family (Thorpe *et al.*, 1997) and the IC family (Gubler *et al.*, 1992).

Near identity of HsdR and HsdM subunits within a family allows bacteria to have two or more R-M systems with different specificities by having several *hsdS* genes

but only single copies of *hsdR* and *hsdM*. HsdR and HsdM can interact with alternative HsdS subunits and form enzymes with different specificities. *Mycoplasma pulmonis* have several *hsdS* genes and a control system regulating their expression so that when some R-M systems are active the others are silent and vice versa (Dybvig *et al.*, 1998). Possibly, the complexity of structure and function of type I systems provides some other advantages including regulatory mechanisms permitting wide distribution of type I R-M systems among bacteria via horizontal gene transfer.

1.1.2. Type II R-M systems.

Type II R-M systems are the simplest of the R-M systems in their structure and function. The finding that type II restriction enzymes cut DNA within or close to their targets made them an exceptionally useful tool for molecular biology and stimulated screening of different bacteria for the presence of type II R-M systems. Hundreds of type II systems are found among a great variety of bacteria.

The restriction and modification activities of type II systems are within two separate enzymes that recognise the same DNA sequence as a target. One is a restriction endonuclease that requires Mg^{2+} as a cofactor and is able to cleave DNA at the target site. The other is a DNA methyltransferase that methylates the recognition sequence and thereby protects it from cleavage by the endonuclease.

Type II recognition sequences are generally symmetrical and can be considered as two identical halves. Many of them are short continuous palindromes of 4, 6 or 8 bp (e.g. GAATTC), some are interrupted by a non-specific spacer of defined length for each system ($CCAN_6TGG$). Methylated residues can be m^5C, m^4C or m^6A, located symmetrically on both strands of the target sequences. Type II methyltransferases are monomers (Rubin & Modrich, 1977; Yoo & Agarwal, 1980; Gunthert *et al.*, 1981) that recognise a complete target and methylate one strand at a time. Type II endonucleases are mostly homodimers and each monomer recognises one half of a target sequence (Kim *et al.*, 1990; Winkler *et al.*, 1993). This is consistent with the idea that each subunit introduces a single-stranded nick into the DNA if both halves are unmethylated. A co-ordinated cleavage of both DNA strands occurs symmetrically and within the recognition sequence. If the nicks are in the centre of the target, i.e. exactly opposite to each other, then a DSB with blunt ends is

generated. If the nicks are separated by several nucleotides then the ends have either 5′ or 3′ overhangs.

Some type II endonucleases have been shown to act on hemimethylated DNA; *Bsp*I and *Sau*3A recognition sites methylated in only one DNA strand are subject to single strand scission within the unmodified strand (Koncz *et al.*, 1978; Streeck, 1980). This finding also supports the idea of structural and functional symmetry of type II restriction enzymes.

Other type II restriction enzymes, e.g. *Eco*RII, *Nae*I, *Sac*II require simultaneous binding of two recognition sites for their activity (Kruger *et al.*, 1988; Conrad & Topal, 1989; Pein *et al.*, 1989). This implies that the interaction between complexes bound to unmodified target sequences is necessary for DNA cleavage. Such interactions might help to avoid autorestriction when rare unmodified targets appear in the resident DNA as a result of DNA repair or under-modification.

Genes specifying type II methylases and their cognate endonucleases are encoded by plasmids (Yoshimori *et al.*, 1972) or located in the chromosome (Chater & Wilde, 1980; Lacks *et al.*, 1986). In both cases they are linked to each other but are transcribed separately, either in the same or in the opposite direction (Wilson & Murray, 1991). Some systems include a third ORF encoding a small protein that has regulatory functions (see 1.2).

Despite the fact that pairs of nucleases and methylases recognise the same DNA sequences as targets, no sequence similarity is found between them. Generally little sequence similarity is found between nucleases with the same specificity but isolated from different bacteria. This finding might imply independent origins not only for a methylase and a nuclease within a type II R-M system but also for different nucleases. All methylases from different systems share regions of similarity (Lauster *et al.*, 1989; Posfai *et al.*, 1989) and this supports a common origin of their functional domains.

Type IIs systems. Type IIs systems (*s*hifted cleavage) are essentially similar to the classical type II systems in their organisation and function but they cleave the DNA at a precise distance of 1 to 20 bp outside their recognition sequences which are asymmetric (Kleid *et al.*, 1976; Brown & Smith, 1977). Their classification as type II systems is based on their simple cofactor requirements (AdoMet for the methylases

and Mg^{2+} for the nucleases) and the fact that methyltransferases and endonucleases are separate enzymes.

The asymmetrical character of the targets requires two different methylases to modify two DNA strands because the recognition sequence in one of them is different from that in the other. Such methylases are found either as two separate enzymes (Lubys *et al.*, 1996) or as a single polypeptide with two rather independent domains each of which can methylate one of the DNA strands (Looney *et al.*, 1989).

Type IIs endonucleases are monomeric in solution (Kaczorowski *et al.*, 1989; Janulaitis *et al.*, 1992; Sektas *et al.*, 1992; Tucholski *et al.*, 1995) and bind DNA as monomers (Sektas *et al.*, 1995; Hirsch *et al.*, 1997). This is relevant to the asymmetrical character of their targets. Recently, however, dimerization was shown to be necessary for *Fok*I endonuclease activity (Bitinaite *et al.*, 1998) and it was suggested that dimerization occurs after the first molecule of the enzyme is bound to a recognition sequence.

1.1.3. Type III systems.

Very few type III R-M systems have been identified and characterised. *Eco*P1 and *Eco*P15I are encoded respectively by the P1 prophage and the p15B plasmid found in *E.coli* (Arber & Dussoix, 1962; Arber & Wauters-Willems, 1970). Many *Salmonella* strains have the chromosomally encoded *Sty*LTI system (Colson *et al.*, 1970; Bullas *et al.*, 1980) and *Hinf*III is found in *Haemophilus influenzae* (Kauc & Piekarowicz, 1978). ORFs that share amino acid similarity to type III systems are identified in the genomes of *Bacillus cereus* and *Helicobacter pylori* (Hegna *et al.*, 1992; Roberts, 1998).

Type III systems are encoded by two genes, *res* and *mod*, transcribed in the same direction but from separate promoters and with *mod* upstream of *res* (Humbelin *et al.*, 1988; De Backer & Colson, 1991a; Sharrocks & Hornby, 1991). Mod is a functional analog of the type I methyltransferase complex M_2S_1. It defines the DNA specificity and possesses methyltransferase activity using AdoMet as a cofactor (Brockes *et al.*, 1972). Like HsdR, Res has no enzymatic activity on its own but confers the endonuclease activity to the ResMod complex. Restriction requires Mg^{2+}

and ATP and is stimulated by AdoMet (Haberman, 1974; Reiser & Yuan, 1977; Kaus & Piekarowicz, 1978; Saha & Rao, 1995).

The recognition sequences of type III enzymes are asymmetric and 5 or 6 bp in length. The particularity of this class of R-M systems is that the methylated residue m^6A is present only in one DNA strand (Bachi *et al.*, 1979; Hadi *et al.*, 1979; Piekarowicz *et al.*, 1981; De Backer & Colson, 1991c; Meisel *et al.*, 1991). For a long time it remained unknown how the enzymes distinguish between modified and newly replicated DNA until it became clear that the orientation of the targets is important (Meisel *et al.*, 1992). DNA with unmodified targets in the same orientation, that is common after DNA replication, is not a substrate for restriction. If unmodified recognition sequences are in the opposite orientation then restriction enzymes initiate ATP-dependent DNA translocation and cleave the DNA on the 3′ side of the recognition sequences at a distance of approximately 25-27 bp (Meisel *et al.*, 1995). Apparently, an interaction of two complexes is required for restriction. The nature of these interactions is unclear. Two complexes of different but closely related type III systems *Eco*P1 and *Eco*P15I can interact as effectively as a pair of the restriction enzymes of either system to cut unmodified DNA (Kunz *et al.*, 1998). Sequences, similar to helicase domains, are found in Res; they might be relevant to the mechanism of translocation which can be similar for type I and type III R-M systems (Dartois *et al.*, 1993; Murray *et al.*, 1993; Meisel *et al.*, 1995).

*Eco*P1 and *Eco*P15I are closely related. Like type I systems from the same family, their subunits are interchangeable and immunologically cross-reactive and recombination generates functional hybrid genes (Arber & Wauters-Willems, 1970). The *res* genes are largely homologous as are the 5′- and 3′-parts of the *mod* genes (Iida *et al.*, 1983; Humbelin *et al.*, 1988). Apparently, the central, dissimilar, regions of the Mod subunits correspond to TRDs although some substitutions localised in this area affect AdoMet binding (Rao *et al.*, 1989). The conservative domains are thought to be involved in subunit-subunit interactions and catalytic activities.

1.1.4. Restriction systems specific for modified DNA

Restriction systems specific for modified DNA consist of only an endonuclease that recognises and cleaves DNA carrying a specific "modification imprint" which is not

characteristic of the host bacterium. Evidence for the existence of this class of systems is found for *Streptococcus pneumoniae* (Lacks & Greenberg, 1977), *Brevibacterium flavum*, *Corynebacterium glutamicum* (Vertes *et al.*, 1993), *Streptomyces* species (MacNeil, 1988) and *E.coli* (Raleigh & Wilson, 1986).

E. coli K-12 specifies three modification-dependent restriction systems encoded by the *mcrA* (*rglA*), *mcrBC* (*rglB*) and *mrr* genes. *mcrA* and *mcrBC* were discovered as factors that affected propagation of T-even phages with nonglycosylated hydroxymethylcytosine (hmC) in their DNA and therefore were called *rglA* and *rglB* (restriction of glucoseless DNA) respectively (Luria & Human, 1952). In the late 1980s it became clear that the Rgl systems are not specific for T-even phages. Rgl affected the cloning in *E. coli* of genes encoding some type II cytosine methyltransferases (Noyer-Weidner *et al.*, 1986; Raleigh & Wilson, 1986). Because cytosine methylation is common for eukaryotes the Rgl systems also affect the cloning of DNA from eukaryotes in *E. coli* (Raleigh *et al.*, 1988; Woodcock *et al.*, 1988). m^5C, common in higher organisms, is critical for restriction by the Rgl systems and it was suggested to rename the latter as Mcr (modified-cytosine restriction) (Raleigh *et al.*, 1991).

McrA is coded by e14, a prophage like genetic element of *E. coli* K-12 (Raleigh *et al.*, 1989) mapped at 26′ of the chromosome (Ravi *et al.*, 1985; Raleigh *et al.*, 1988). e14 can be induced by UV irradiation and consequently lost during cell division as a non-replicating circle (Hiom & Sedgwick, 1991; Kelleher & Raleigh; 1994). The e14 DNA encoding McrA was cloned and sequenced to reveal a single ORF with a predicted gene product of 31 kDa (Hiom & Sedgwick, 1991). McrA is active on DNA methylated by the *Hpa*II or *Sss*I cytosine methyltransferases (Hiom & Sedgwick, 1991; Kelleher & Raleigh, 1991).

McrBC is the best investigated modification-dependent system. It is encoded by two genes, *mcrB* and *mcrC*, that form an operon (Ross *et al.*, 1987, 1989a, 1989b). *mcrB* codes for two polypeptides, $McrB_L$ and $McrB_S$; the smaller polypeptide ($McrB_S$) is produced from an in-frame internal translational start site in *mcrB* (Ross *et al.*, 1987, 1989a; Kruger *et al.*, 1992). The $McrB_S$ sequence is identical to that of $McrB_L$ except that it lacks 161 amino acids present in the N-terminus of the latter protein (Ross *et al.*, 1989a).

DNA cleavage *in vitro* requires $McrB_L$, McrC, GTP and Mg^{2+} but not $McrB_S$ (Sutherland *et al.*, 1992). The efficiency of restriction is dependent on the molar ratios of the *mcrBC* gene products (Beary *et al.*, 1997; Panne *et al.*, 1998). It is optimal *in vitro* when $McrB_L$ is in 3 to 5-fold excess over McrC (Panne *et al.*, 1998). *In vivo*, $McrB_S$ may play a role in the sequestration of McrC to provide the optimal ratio of $McrB_L$ to McrC (Beary *et al.*, 1997; Panne *et al.*, 1998). Presumably, the complex of $McrB_S$ with McrC is not functional because the DNA-binding domain resides in a fragment comprising the N-terminal 190 amino acids of $McrB_L$ (Gast *et al.*, 1997) and apparently $McrB_S$ cannot participate in DNA binding whereas $McrB_L$ can (Krüger *et al.*, 1995).

McrBC restricts DNA containing m^5C, m^4C and hmC if either purine precedes a modified cytosine (Blumenthal *et al.*, 1985; Noyer-Weidner *et al.*, 1986; Raleigh & Wilson, 1986). DNA cleavage requires at least two target sites that are optimally separated by 40-80 bp, but can be up to 3 kb apart (Sutherland *et al.*, 1992; Stewart & Raleigh, 1998). The mechanism of restriction is probably similar to those for type I and III R-M systems; when two complexes translocating the same molecule of DNA collide, a DSB is produced in the site of collision and therefore away from recognition sites. While type I and III R-M systems translocate DNA in an ATP-dependent manner, the translocation by McrBC is supported by GTP (Sutherland *et al.*, 1992; Pieper *et al.*, 1997; Panne *et al.*, 1999). A GTP-binding motif is identified in the central part of $McrB_L$ (Dila *et al.*, 1990) and $McrB_L$ binds and hydrolyses GTP in an McrC-dependent fashion (Pieper *et al.*, 1997). McrBC is the only known nuclease that requires GTP for activity.

The Mrr (methylated adenine recognition and restriction) system was discovered as a factor involved in the induction of SOS response in *E. coli* cells expressing *Hha*II or *Pst*I adenine methyltransferases (Heitman & Model, 1987). Later it was found that Mrr also has an effect on m^5C containing DNA; mammalian DNA rich in m^5CG sequences was restricted in *mcrA mcrBC* background (Kelleher & Raleigh, 1991; Kretz *et al.*, 1991).

The *mrr* and *mcrBC* loci surround the *hsd* region in the chromosome of *E. coli* K-12. The *mrr* promoter and the part of the coding sequence of the N-terminal part of the protein overlap with the promoter region of *hsdR*. The *mcrBC* genes are downstream

of the *hsdMS* operon. The three closely linked restriction systems are referred to as an "immigration control region" (Raleigh *et al.*, 1989; Kelleher & Raleigh, 1991).

1.2. Regulation of enzymatic activities of R-M systems

1.2.1. Establishment, maintenance and loss of R-M systems

Cells having an endonuclease capable of generating DSBs must have an appropriate protection of their chromosomal DNA to avoid it being attacked by the nuclease. For R-M systems this can be achieved by maintaining chromosomal target sequences in a methylated state. Replication of fully methylated DNA results in hemimethylated DNA which is methylated by the modification enzyme before the next round of replication. Methylation of hemimethylated targets before replication avoids the appearance of unmethylated DNA which would be a substrate for a restriction enzyme.

When genes specifying an R-M system are transferred to a naive bacterium with an unmethylated chromosome the cell will survive only if its chromosome becomes methylated before the endonuclease appears in the cell. Some type II systems and one type III R-M system are difficult to clone in *E. coli* unless the M (*mod* for type III) gene is introduced first and the chromosome becomes protected by methylation before R (*res* for type III) is transferred to the modification-proficient cells to establish an R-M system. This two step procedure was used to clone *Sty*LTI, a chromosomally encoded type III system (de Backer & Colson, 1991a, 1991b), and some type II systems, e.g. *Dde*I and *Bam*HI (Howard *et al.*, 1986; Brooks *et al.*, 1989).

Genes specifying numerous R-M systems of different types can be cloned in one step. These data imply the existence of regulatory mechanisms effective in *E. coli* which prevent the lethal effect of the acquisition of genes specifying a new R-M system. Such mechanisms were proposed for some type II systems. Type II methyltransferases are active as monomers while endonucleases form dimers. The requirement for the subunit assembly might lead to a lag in the production of restriction enzyme that can be used by the methylase to modify the chromosome before the active nuclease is produced. This mechanism may be common to all type

II systems. In addition, the subunit assembly of the *Pvu*II endonuclease might be influenced by an additional protein encoded by a small ORF within but complementary to the sequence coding the methylase (Adams & Blumenthal, 1995). The predicted peptide of 28 amino acids resembles a region of the *Pvu*II nuclease at the dimer interface. The expression of the ORF leads to a drop in the restriction activity. It was suggested that interaction of the small peptides with the nuclease monomer affects the formation of the active restriction enzyme and delays production of the nuclease. However, no direct experiments on the role of the assembly of endonucleases in the establishment of type II R-M systems have been done.

Another mechanism of control of type II systems is based on transcriptional regulation of the expression of R and M genes. Promoter regions of the R and M genes of *Sso*II, a type II R-M system, overlap each other. The N-terminal 72 amino acids of *Sso*II methyltransferase, predicted to form a helix-turn-helix motif, are responsible for the specific binding of DNA in the promoter region. When the methylase is bound to the operator it represses its own synthesis but stimulates the expression of the cognate restriction endonuclease (Karyagina *et al.*, 1997). Transfer of both genes to a naïve cell will result in the early expression of the M gene; the transcription of R will be dependent on the binding of the methylase to the promoter region to stimulate transcription. As long as unmodified targets are present on the chromosome they will compete with the operator sequence for the binding of the methylase. Therefore the expression of the nuclease will be delayed until the amount of the methyltransferase accumulated is sufficient to methylate most chromosomal targets and bind the operator. A similar mechanism of autogenous regulation was proposed for the *Eco*RII and *Msp*I systems (Karyagina *et al.*, 1997).

Some type II systems have an additional protein, C for *c*ontrol, that plays a regulatory role similar to that for the *Sso*II methylase. This control element is a short polypeptide of approximately 80 amino acids which contains a helix-turn-helix DNA-binding motif. It is encoded by a gene located upstream of R. Like the *Sso*II methyltransferase, it inhibits transcription of M but stimulates expression of R due to its binding to the operator sequences of these genes (Tao *et al.*, 1991; Tao & Blumenthal, 1992; Ives *et al.*, 1992, Rimseliene *et al.*, 1995; Nakayama &

Kobayashi, 1998). In the absence of C the methylase activity is increased 15-fold whereas the endonuclease activity is decreased 100-fold (Ives *et al.*, 1992, 1995). It was suggested that C is critical for the establishment of type II R-M systems (Tao *et al.*, 1991). During the establishment, when C is still not accumulated, the methylase is produced in greater amount than the nuclease and this allows modification of host DNA before the active restriction enzyme appears in the cell. If C is expressed prior to the transfer of M and R then cells do not survive (Nakayama & Kobayashi, 1998) because the presence of C favours the expression of R over M and therefore the endonuclease is synthesised before bacteria complete the methylation of their DNA. Some heterologous pairs of C genes are interchangeable (Ives *et al.*, 1995). As a result, the resident R-M system prevents establishment of an R-M system having the same C specificity but different sequence specificity (Nakayama & Kobayashi, 1998) since the control protein C of the resident system affects the establishment of the new specificity in the same way as the expression of the C gene before the transfer of R-M genes.

Once established type II systems may become obligatory to their host, loss of genes specifying a type II system can cause cell death (Kulakauskas *et al.*, 1995; Naito *et al.*, 1995). When the R and M genes are eliminated, bacteria face the problem of protecting their DNA from residual nuclease. Both methylase and nuclease are diluted along with the bacterial growth and cell division and eventually the amount of methylase is not enough to modify the chromosome completely. If the nuclease is still present in the cell then the chromosome can be cleaved. When R and M genes are cloned in a plasmid they enhance plasmid segregation stability because cells that have lost the plasmid are unable to modify a sufficient number of recognition sites in their chromosomes to protect them from lethal attack by the remaining restriction enzyme molecules. The same mechanism that ensures the stability of type II R-M genes on a plasmid should also enforce the retention and functional integrity of the genes when they are chromosomally encoded. It was proposed to regard a pair of R-M genes as a parasite, symbiont, or "selfish" unit that enforces its retention in hosts whose chromosomes bear the specific sites it recognises (Naito *et al.*, 1995). A mutation in the C gene of *Eco*RV, which probably perturbs the ratio of nuclease and methylase in the favour of the latter, as shown for *Bam*HI (Ives *et al.*, 1992),

eliminates the stabilisation effect of R-M system through postsegregational killing of the host (Nakayama & Kobayashi, 1998).

Genes specifying type I and most type III R-M systems can be transferred readily from one strain to another in one step by conjugation, transformation or P1 transduction (Boyer, 1964; Glover & Colson, 1965; Colson & Colson, 1972; Arber, 1974; Sain & Murray, 1980; Bullas *et al.*, 1980; Fuller-Pace *et al.*, 1985; Suri & Bickle, 1985; Redaschi & Bickle, 1996b; Kulik & Bickle, 1996). Experiments, quantifying such a transfer for type IA systems, have found no evidence for cell death (O'Neill *et al.*, 1997). Early studies with phage P1 R-M activities (*Eco*P1, a type III system) demonstrated that the modification activity is already detectable a few minutes after infection of *E.coli* with P1 whereas restriction activity attains its normal level only some hours later (Arber, 1974). In relatively recent experiments for some R-M systems of type I and III, the expression of the restriction-proficient phenotype has been shown to require up to 15 generations after the R-M genes enter naive cells (Prakash-Cheng & Ryu, 1993; Kulik & Bickle, 1996; Redaschi & Bickle, 1996b).

Although *hsdR* and *hsdMS* for type I are transcribed from different promoters, p_{res} and p_{mod} respectively (Sain & Murray, 1980), the effective regulation at a transcriptional level has not been detected for the systems investigated. *lacZ*-fusions with promoters of $hsdR_K$ and $hsdMS_K$ reveal no difference in β-galactosidase expression after these fusions are transferred to unmodified recipients by conjugation (Prakash-Cheng *et al.*, 1993). Promoter assays for *Eco*R124I (IC family) show that p_{mod} is slightly stronger than p_{res} but both promoters are very weak (Kulik & Bickle, 1996). Possibly, even this small difference in the strength of the promoters under conditions of weak transcription could result in the delayed expression of the nuclease activity. However, the finding for *Eco*R124I that the expression of *hsdR* prior to the transfer of *hsdMS* does not lead to cell death implies additional mechanisms of regulation that can be provided either by plasmid-encoded functions or by bacterial ones, or by both.

For *Eco*P1 and *Eco*P15I (type III) it has been shown that the methyltransferase affects the amount of the endonuclease in the cell (Redaschi & Bickle, 1996). Very little Res is detected by Western blots when *res* is expressed from a multicopy

plasmid in the absence of *mod*. Rapid proteolysis of Res in the absence of Mod is suggested to explain the low concentration of Res in *mod*$^-$ cells. However, this amount of Res is enough to kill *res*$^+$*mod*$^-$ cells after the *mod*$^+$ allele had been transferred to the cells to produce active nuclease. A similar killing effect is a characteristic of the type IA and IB families (Fuller-Pace *et al.*, 1985; Suri & Bickle, 1985; Kelleher *et al.*, 1991). *hsdR*$^+$*M*$^-$*S*$^-$ cells expressing the gene from a multicopy plasmid die upon the acquisition of *hsdM*$^+$*S*$^+$. *Eco*R124I behaves differently and over-expression of *hsdR* prior to the transfer of *hsdM*$^+$*S*$^+$ is not lethal for the recipient cell (Kulik & Bickle, 1996). The distinction in the behaviour of type IA, IB and III systems from that of a type IC system might reflect the difference in regulation mechanisms involved in the establishment of R-M systems.

It has been found that for type IA and IB systems a host factor is involved in the control of restriction activity following conjugation. A derivative of *E. coli* C, *hsdC,* incapable of the establishment of the *Eco*KI system was isolated (Prakash-Cheng *et al.,* 1993). Conjugative transfer of F'*hsd*$_K$*R*$^+$*M*$^+$*S*$^+$ to this mutant occurs with a frequency 10^5 lower in comparison with F'*hsd*$_K$*R*$^-$*M*$^+$*S*$^+$, presumably because the cells are killed following the entry of the *hsd*$^+$ plasmid and the consequent production of the endonuclease while the resident chromosome is still unmodified. The lethal effect of F'*hsd*$_K$*R*$^+$*M*$^+$*S*$^+$ transfer for *hsdC* cells is confirmed by a drop in the titre of the recipient bacteria during conjugation.

Low efficiency of transfer of type IA *hsd* genes to an *hsdC* mutant has been shown by P1 transduction experiments (O'Neill *et al.*, 1997). The frequency of cotransduction of *dnaC-hsd* in *E. coli* K-12 is about 50%. However no $r_D^+m_D^+$ recombinant among 100 *dnaC*$^+$ were obtained for an *hsdC* recipient while the wild-type *E. coli* C revealed about 20% linkage. Similar cotransduction frequencies between DnaC$^+$ and m_D^+ are observed for the transfer of *hsd*$_D$*R*$^-$*M*$^+$*S*$^+$ irrespective of the *hsdC* allele.

Conjugative transfer of the *hsd* genes of the *Eco*AI system (type IB) leads to results similar to those for *Eco*KI (Kulik & Bickle, 1996). A decrease in the frequency of F'*hsd*$_A$*R*$^+$*M*$^+$*S*$^+$ transfer and recipient killing have been observed. However, both restriction and modification activities are found in transconjugants. No difference in the level of restriction in *hsdC*$^+$ and *hsdC*$^-$ cells of *E.coli* C has been shown for either

*Eco*AI (Kulik & Bickle, 1996) or *Eco*KI (N.E.Murray, pers. commun.). HsdC seems to be a modulator of the restriction activity of members of the type IA and IB families at least at the stage of establishment, but not for type IC, since hsd^{+}_{R124I} can be transferred easily to an *hsdC* recipient (Kulik & Bickle, 1996).

The complex structures of type III and especially type I endonucleases allow regulation of their activity based on different affinities with which subunits bind to each other. Analysis of the *in vitro* assembly pathway of the *Eco*KI endonuclease reveals more than just two (M_2S_1 and $R_2M_2S_1$) complexes (Dryden *et al.*, 1997). However, only $R_2M_2S_1$ exhibited endonuclease activity, whereas interaction of R with M_1S_1 leads to a non-functional protein $R_1M_1S_1$. It was suggested that M_1S_1 at higher concentration than M_2S_1 might subvert R subunits and prevent their binding to M_2S_1. According to this idea, HsdC may either degrade inactive complexes $R_1M_1S_1$, M_1S_1 and free R and M subunits or bind R to prevent its interaction with M_2S_1.

The $R_2M_2S_1$ complex is formed in two steps. Binding of the first R-subunit to methylase M_2S_1 results in $R_1M_2S_1$ protein which can interact with another R-polypeptide to establish an active nuclease $R_2M_2S_1$. Binding of both R-subunits for type IA systems occurs with the same affinity (Powell *et al.*, 1998) whereas type IC methyltransferases bind the first molecule of HsdR with much greater affinity than the second one (Janscak *et al.*, 1998). This particularity of type IC systems might have a positive effect on the bacterial survival during the establishment of a new specificity because the active nuclease $R_2M_2S_1$ could appear in the cell only after most of the methylase complexes have bound the first R-subunit. The time necessary for the saturation of the methylase with the first R-polypeptide might provide a delay in the expression of the endonuclease activity over methylation.

Unlike type II systems, type I systems do not display "selfish" behaviour. Early genetic experiments demonstrated that about 50% of random mutations that affect R-M systems abolish both restriction and modification, i.e. lead to a loss of specificity (Wood, 1966). Replacement of one R-M system with a system with a different specificity, accompanied by both the establishment of the new specificity and by the loss of the old one, can be done easily via conjugation or P1 transduction (Glover & Colson, 1965). After the selfishness of type II systems had been discovered, O'Neill *et al.* (1997) have shown that the hsd_K^{+} genes cloned in a plasmid do not stabilise the

maintenance of the replicon and can be readily lost by the cells. Similarly, the loss of the chromosomal hsd_K^+ genes as the result of P1 transduction mediated substitution of the functional allele with *Δhsd* does not influence the bacterial survival, even in the case of *hsdC* cells (O'Neill *et al.*, 1997).

Some experiments suggest that hsd^+ bacteria contain more *Eco*KI methyltransferase M_2S_1 than endonuclease $R_2M_2S_1$ (Weiserova *et al.*, 1993; Webb *et al.*, 1996). This might explain bacterial survival following the loss of a type I specificity. By the time the nuclease is diluted out the remaining amount of the methylase is sufficient to methylate the chromosome. In this case no additional regulation is required to prevent cell death. However, this explanation raises another question. It is not clear why the methylase is in excess over the nuclease although both promoters (p_{res} and p_{mod}) are equally active (Loenen *et al.*, 1987; Prakash-Cheng *et al.*, 1993) and one might expect equimolar amount of all three subunits, or at least R and M, to be produced. One cannot exclude the possibility that regulation at the level of mRNA stability, initiation of translation or protein stability might affect the concentrations of the subunits in bacteria. None of these possibilities has been tested although the experiments by Weiserova *at al.* (1993) suggest that the rate of synthesis of HsdM and HsdS for *Eco*KI is higher than that for HsdR.

1.2.2. Restriction alleviation

The phenomenon of restriction can be observed following the infection of bacteria with foreign DNA that enters cells via conjugation and transformation or injected by phage. If the DNA is not methylated at target sequences recognised by a bacterial R-M system it becomes a substrate for degradation by a restriction enzyme. The efficiency of restriction can be influenced by the physiological state of the bacterium at the time of infection and, under some conditions, genotypically restriction-proficient bacteria show a drop in restriction referred to as restriction alleviation (RA). On the other hand, some phages and plasmids encode antirestriction functions that allow them to overcome restriction. In these cases alleviated restriction is not relevant to the regulation of resident restriction and modification systems by host bacteria.

Host-controlled restriction alleviation. RA of a type I system was first documented as early as 1953 when Bertani and Weigle found that UV-irradiation of wild-type *E.coli* prior to infection partially relieved the restriction of λ.C, i.e. unmodified λ (Bertani & Weigle, 1953). Within the next 20 years many different phenomena such as induction of lytic phage in strains carrying an inducible phage, increased repair and mutagenesis of UV-irradiated phage, filament formation, increased frequency of mutations were found to be associated with UV irradiation of bacteria. It was suggested that UV irradiation derepresses the production of new proteins that are responsible for these phenomena (Defais *et al.*, 1971; Radman, 1975), i.e. activates the SOS response. The hypothetical mechanism was shown to be dependent on RecA and LexA (Miura & Tomizawa, 1968; Defais *et al.*, 1971; McPartland *et al.*, 1980). UV-induced RA (UV-RA), like any other SOS-induced function, does not occur in *recA* and *lexA* (Ind$^-$) bacteria (Day, 1977; Hiom & Sedgwick, 1992; Kelleher & Raleigh, 1994). The SOS response can be activated by treatments that block replication, e.g. sub-lethal concentrations of nalidixic acid present in growth media (Cowlishaw & Ginoza, 1970; Gudas & Pardee, 1976; McPartland *et al.*, 1980). Such treatment also induces RA of type I systems in a RecA- and LexA-dependent manner (Thoms & Wackernagel, 1984). Both UV-RA and Nal-RA require functional RecBC (Dharmalingam & Goldberg, 1980; Thoms & Wackernagel, 1982, 1984) although the protein is necessary for derepression of RecA and the consequent SOS induction only in response to nalidixic acid (Gudas & Pardee, 1976) but not after UV irradiation of cells (Bockrath & Hanawalt, 1980; McPartland *et al.*, 1980) and one might expect UV-RA to be independent of RecBC. Derepression of RecA following UV irradiation occurs in a RecF-dependent manner (McPartland *et al.*, 1980; Karu & Belk, 1982) and *recF* mutants are deficient in UV-RA (Thoms & Wackernagel, 1984; Kelleher & Raleigh, 1994) but not in Nal-RA (Thoms & Wackernagel, 1984). In summary, activation of the SOS response seems obligatory for the induction of RA by UV light or nalidixic acid.

Does the SOS response lead to the derepression of synthesis of a protein necessary for RA? If chloramphenicol is added following UV irradiation of bacteria to stop protein synthesis then RA is blocked (Thoms & Wackernagel, 1984; Kelleher & Raleigh, 1994). It is logical to expect that the function necessary for RA is a member

of the SOS regulon controlled by LexA. Indeed, in *umuD* and *umuC* mutants UV-RA is 100-fold lower than in *umu*$^+$ bacteria (Hiom & Sedgwick, 1992). UmuD′$_2$C is a key component of the SOS-activated mutagenesis which enables the DNA replication machinery to pass damaged template via an error-prone mechanism (Lawrence *et al.*, 1990; Rajagopalan *et al.*, 1992; Koffel-Schwartz *et al.*, 1996). Recently it has been shown that UmuD′$_2$C is an independent DNA polymerase (Tang *et al.*, 1999) whose expression is regulated so tightly that the active complex appears in cells after other, proof-reading, mechanisms of DNA repair have been used, thereby minimising the error-prone replication (Opperman *et al.*, 1999). The *umuDC* operon is controlled by LexA and it is derepressed during the SOS response. In addition, activated RecA is necessary to stimulate autocleavage of UmuD to produce UmuD′ (Burckhardt *et al.*, 1988; Shinagewa *et al.*, 1988). A mutation in *umuD* that results in a non-cleavable form of UmuD leads to a 100-fold reduction of RA (Hiom & Sedgwick, 1992).

A mutation in *recA* (*recA730*) that leads to constitutive derepression of the SOS regulon does not cause relief of restriction. Neither does the constitutive expression of the cleaved UmuD′ proteolytic fragment (Hiom & Sedgwick, 1992) therefore the SOS induction is not sufficient for RA, the presence of DNA lesions is also necessary.

RA is a transient process gradually developing after an SOS-inducing treatment (Thoms & Wackernagel, 1984; Hiom & Sedgwick, 1992; Kelleher & Raleigh, 1994). It reaches its peak 1-2.5h after treatment and efficient restriction is restored afterwards. Vigorous aeration following UV-irradiation was mentioned as a factor stimulating RA (Hiom & Sedgwick, 1992).

RecBC is necessary in the early stages of the development of the RA phenotype (Thoms & Wackernagel, 1984). In a *recBC sbcB* mutant the recombination and repair proficiencies are restored (Kushner *et al.*, 1971) as well as the inducibility of RecA synthesis by nalidixic acid (Karu & Belk, 1982). However, the suppresor mutation does not derepress the block of either UV-RA or Nal-RA in *recBC* cells (Thoms & Wackernagel, 1984; Kelleher & Raleigh, 1994). The other suppressor of the recombination-deficiency of *recBC* bacteria, *sbcA*, causes constitutive RA. In the absence of any treatment a *recBC sbcA* mutant restricts 100-fold less well than

recBC cells, probably because of derepression of the antirestriction function *lar* of the Rac prophage (Simmon & Lederberg, 1972; Thoms & Wackernagel, 1982).

Constitutive RA also is a characteristic of *dam* mutants that restrict about 100-fold less well than dam^+ bacteria (Efimova *et al.*, 1988a). The *dam* gene of *E.coli* encodes a DNA methyltransferase which methylates GATC sequences in double-stranded DNA. Dam-methylation allows mismatch repair to discriminate between parental and daughter strands and to operate on a newly synthesised strand carrying replication errors (Wagner & Meselson, 1976; Pukkila *et al.*, 1983). Uncoordinated mismatch repair in *dam* mutants leads to DSBs (Wang & Smith, 1986). The SOS induction is constitutive in *dam* mutants (Peterson *et al.*, 1985) and might be caused by DSBs. However, mutations in *mutH*, *mutL*, or *mutS* genes that prevent DSBs do not supress either the constitutive SOS induction or the RA (Peterson & Mount, 1993; Kelleher & Raleigh, 1994). UV-RA does not influence modification (Hiom & Sedgwick, 1992) whereas the deficiency in *dam* affects not only restriction but also the methyltransferase activity of *Eco*KI, and so, modification is decreased in *dam* strains (Efimova *et al.*, 1988a).

RA can be induced in response to 2-aminopurine (2-AP) and 5-bromouracil (5-BU) (Efimova *et al.*, 1988b) but this kind of RA differs from UV-RA and Nal-RA since it does not require either functional RecA or LexA. RA induced by 2-AP can be blocked by chloramphenicol or nalidixic acid and it was suggested that DNA replication is necessary for RA. The role of protein synthesis is unclear because the initiation of the chromosome replication is known to require *de novo* protein synthesis (Ward & Glaser, 1969) and therefore chloramphenicol might have a dual effect by blocking both protein synthesis and replication. 2-AP and 5-BU are mismatch inducing agents and it was suggested that mismatches function as inducing signals for RA (Efimova *et al.*, 1988b). This idea is supported by the finding that mutations in *mutH*, *mutL* or *mutS*, genes that abolish the repair of naturally occurring mismatches, lead to a 5-fold relief of restriction in untreated cells (Efimova *et al.*, 1988b).

Neither type II nor type III systems show RA in response to 2-AP or UV irradiation (Thoms & Wackernagel, 1982; Efimova *et al.*, 1988b). RA seems to be a characteristic of type I R-M systems, and possibly of some restriction systems

specific for modified DNA. The restriction activity of one such system, McrBC, is alleviated in response to UV irradiation (Dharmalingam & Goldberg, 1980; Kelleher & Raleigh, 1994). UV-RA of McrBC resembles UV-RA for type I R-M systems in its dependence on functional *recA, lexA* and *recF* (Dharmalingam & Goldberg, 1980; Kelleher & Raleigh, 1994). The results concerning the involvment of RecBC in the process are contradictory: Dharmalingam and Goldberg (1980) reported that UV-RA did not occur in *recBC sbcB* cells whereas Kelleher and Raleigh (1994) observed a 180-fold drop of restriction by UV irradiated *recBC sbcB* bacteria. This disagreement might be due to differences in the experimental conditions.

McrA, another *E.coli* K-12 restriction system that cleaves specifically modified DNA, is encoded by a defective prophage e14 (Brody *et al.*, 1985; Raleigh *et al.*, 1989). UV irradiation induces prophage excision from the chromosome followed by its loss during cell division (Brody *et al.*, 1985; Hiom *et al.*, 1991; Kelleher & Raleigh, 1994). UV treatment, therefore, causes irreversible loss of McrA restriction proficiency and this phenomenon cannot be considered as an example of RA.

Mrr is the third *E. coli* K-12 restriction enzyme specific for methylated DNA. Its activity was reduced 10-fold in response to UV irradiation in a RecA- and LexA-independent manner (Kelleher & Raleigh, 1994).

The mechanisms of RA remain unclear. Three general pathways by which RA might be accomplished were suggested by Kelleher & Raleigh (1994): "cells displaying RA could simply express less of the enzyme; cells displaying RA could express a protein that degrades, inhibits, or competes with the restriction enzyme; or cells displaying RA could contain a product of repair – a DNA site or structure, or a metabolite – that depletes the cell of the restriction enzyme, rendering it unavailable for action on incoming DNA."

The pathways for RA of different systems may be similar. The dependence of UV-RA of both *Eco*KI and McrBC on *recA, lexA* and possibly *recBCD* supports this idea. In this case, when two (or more) systems are present in a cell they may affect RA of each other by competing for unknown factors necessary for RA and RA of one system would derepress the development of RA of the other one. Alternatively, the mechanism of RA of one system might sequester a factor which normally provides a high level of restriction by the other system. Then RA of one system could indirectly

stimulate RA of the other. Such effects can be avoided by having a single restriction system per strain used for RA experiments.

Antirestriction proteins. Restriction systems are thought to have evolved to defend bacteria against incoming DNA such as plasmids and bacteriophages. However, some plasmids and phages developed functions that allow them to overcome restriction. The mechanisms of overcoming restriction barriers are very different (see Kruger & Bickle, 1983; Bickle & Kruger, 1993). Some of them are based on a specific modification of DNA, e.g. methylation, acetimidation, or glucosylation that block the recognition of targets by restriction enzymes, or changes in DNA that lead to the loss of DNA sequences recognised by host restriction systems in phage genome. Others rely on proteins which are encoded by invading agents and modulate either restriction or modification activities or both. This modulation results in poorer restriction that consequently has been called restriction alleviation.

The lambdoid phages λ, ϕ21 and P22 encode a protein, Ral, that affects type IA R-M systems (Zabeau *et al.*, 1980; Franklin, 1985; Loenen & Murray, 1986; Semerjian *et al.*, 1989). Both λ*ral*$^+$ and λ*ral*$^-$ (*ral* stands for *r*estriction *al*leviation) unmodified phages are restricted efficiently by a type IA system but if they escape restriction or propagate on an r^-m^+ host then the progeny of the λ*ral*$^+$ phages are fully modified whereas those for λ*ral*$^-$ are poorly methylated (Zabeau *et al.*, 1980). Ral alleviates restriction by changing the activity of the *Eco*KI methylase. In the presence of Ral the methylase modifies unmethylated DNA as efficiently as hemimethylated (Loenen & Murray, 1986) and therefore type I systems become similar to type IB enzymes.

A derivative of phage λ, λ*reverse* (λ*rev*) (Zissler *et al.*, 1971) expresses a Ral-like activity, Lar, (Simmon & Lederberg, 1972; Toothman, 1981) encoded by the *lar* gene (Toothman *et al.*, 1981). λ*rev* phages arise by recombination between phage λ and the excised Rac prophage (Kaiser & Murray, 1979). The Rac prophage is a defective lambdoid prophage in the chromosome of many *E. coli* strains (Kaiser & Murray, 1979). It includes genes for integration, recombination, immunity and replication (Low, 1973; Gottesman *et al.*, 1974; Diaz *et al.*, 1979) analogous to those of the λ phage. These functions are normally repressed but can be activated when the prophage excises from the chromosome or as the result of a mutation. *E. coli sbcA* mutants express the recombination genes *recE* and *recT* (Toothman, 1981; Clark *et*

al., 1993). They are also phenotypically Lar$^+$ (Simmon & Lederberg, 1972). The effect of Lar can be seen even in the absence of the *sbcA* mutation. *lar*$^+$ (*rac*$^+$) strains restrict λ.0 10-fold less well than *lar*$^-$ (*rac*$^-$) bacteria (Loenen & Murray, 1986).

Phage P1 antirestriction system is encoded by two genes *darA* and *darB* (Iida *et al.*, 1987; Streiff *et al.*, 1987). The Dar proteins are synthesised during the late stage of P1 infection and packaged in the phage head along with DNA (Iida *et al.*, 1987). During infection the Dar proteins penetrate into cells along with injected DNA and protect it from restriction by type I systems. Both DarA and DarB are required for antirestriction against type IA systems whereas DarA is sufficient to overcome restriction by type IB systems (Iida *et al.*, 1987). It is suggested that the Dar proteins bind DNA in a phage head and remain bound to the DNA after injection. This binding is non-specific because any DNA packaged in a P1 phage head becomes protected. This allows efficient generalised transduction between hosts with different type I R-M systems (Iida *et al.*, 1987). Dar-mediated protection does not work *in trans*. Co-infection of Dar$^+$ unmodified phage with a Dar$^-$ unmodified phage does not protect the latter from restriction. In addition, the Dar system stimulates modification. Dar$^-$ phage have less than 1% of the modification of Dar$^+$ phage after a single cycle of growth on a restriction-deficient and modification-proficient host (Iida *et al.*, 1987).

The antirestriction systems encoded by closely related phages T3 and T7 are relatively well understood. Phage 0.3 (*ocr*) gene codes for 0.3 or Ocr protein that specifically binds to type I restriction enzymes and blocks both the endonuclease and methylase activities (Spoerel *et al.*, 1979; Bandyopadhyay *et al.*, 1985; Kruger *et al.*, 1985). The *ocr* gene is located at the left end of the phage genome, the end injected into the cell first. The expression of *ocr* precedes the penetration of the rest of the phage genome, which contains multiple target sequences for type I systems, and prevents its restriction. The T3 (but not T7) Ocr possesses AdoMet hydrolase activity (Studier & Movva, 1976). As a result, it has an inhibitory effect on not only type I but also on type III restriction enzymes which require AdoMet for their nuclease activity (Haberman, 1974; Reiser & Yuan, 1977).

Restriction is likely to be encountered as a barrier to transfer of plasmids between bacterial strains in nature. Like phages, some plasmids have evolved antirestriction

functions. Genes specifying the *ardA* antirestriction systems are identified among plasmids of 5 out of 23 incompatibility groups tested (Chilley & Wilkins, 1995). Like *ocr*, *ardA* genes are located in leading regions of plasmids and therefore during conjugation they are transferred to recipients early (Delver *et al.*, 1991; Belogurov *et al.*, 1992) and probably transcribed only from ssDNA (Althorpe *et al.*, 1999). Such transcription could confine the production of ArdA during conjugation to the period before the plasmid DNA becomes double stranded. ArdA of the ColIb-P9 plasmid (IncI1 group) acts specifically against type I R-M systems (Delver *et al.*, 1991; Read *et al.*, 1992) and alleviates DNA restriction. Over-expression of *ardA* from a multicopy plasmid also impairs host-controlled modification activity (Read *et al.*, 1992; Belogurov *et al.*, 1993). ArdB and ArsR are two other plasmid-encoded antirestriction proteins active on type I systems (Belogurov *et al.*, 1993; Rastorguev *et al.*, 1998). Unlike ArdA, ArdB does not influence modification activity. ArdB and ArsR are not similar to ArdA except for a short 9 amino acid motif found in different antirestriction proteins and suggested to be an interaction site for antirestriction proteins with restriction endonucleases (Rastorguev *et al.*, 1998).

1.3. Proteolysis as a mechanism of regulation of cellular processes in *E. coli*.

Very few examples of proteolysis-dependent regulation of bacterial functions have been known until recently. Most known *E. coli* proteases have been identified in the last decade and the data on their involvement in different regulatory pathways are expanding. The role of proteolysis is even broader in *Bacillus* and *Caulobacter*, bacteria with more complex life cycles, where switches of developmental pathways are based on proteolysis-dependent regulation. All the studies on the structure and enzymology of prokaryotic proteases have been done from those of *E. coli*. Highly conserved homologs of many *E. coli* proteases are present in other bacteria and eukaryotic organelles. It appears that proteolysis is a regulatory mechanism common to both prokaryotes and eukaryotes and even some viruses whose life cycle depends on host proteases.

1.3.1. Cytoplasmic proteases: structure and substrates

Lon, ClpAP, ClpXP, ClpYQ (HslUV) and HflB (FtsH) are cytoplasmic proteases identified in *E. coli*. They are organised as multisubunit assemblies with ATPase and peptidase activities that are localised either in different polypeptides or in the same subunit. As a result, many exist as hetero-oligomers with two types of subunits (ClpAP, ClpXP, ClpYQ) whereas others exist as homo-oligomers (Lon and HflB). Because proteolysis became a subject of intensive investigations only a few years ago a relatively small number of proteins have been identified as substrates for these proteases and they will be mentioned below.

ClpAP and ClpXP. ClpP and ClpX are encoded by the *clpPX* operon that is under the control of the standard sigma factor, σ^{70}, and the heat shock sigma σ^{32} (Kroh & Simon, 1990). *clpA* is a separate gene whose expression is independent of σ^{32} (Katayama *et al.*, 1988).

ClpP is synthesised as a precursor of 23 kDa that is autoproteolysed to produce an active form of 21 kDa (Maurizi *et al.*, 1990a, 1990b). Fourteen ClpP polypeptides are arranged in two heptameric rings stacked on to each other so that they form a cavity with 14 peptidase catalytic sites located within the cavity (Maurizi *et al.*, 1990a, Flanagan *et al.*, 1995; Kessel *et al.*, 1995; Wang *et al.*, 1997), thereby protecting cytoplasmic proteins from indiscriminate degradation. Axial pores, only large enough to allow access by small polypeptides and unfolded proteins, are seen at either end of ClpP and presumed to be the sites where substrates enter. Possibly, because of the size of the pores, the ClpP multisubunit complex possesses only peptidase (but not protease) activity, which is dependent on the active serine, the key residue in the catalytic centre (Maurizi *et al.*, 1990b).

ClpA and ClpX are ATPase components of the appropriate proteases (Katayama *et al.*, 1988; Maurizi *et al.*, 1990a; Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993). ClpA has two ATP-binding sites (Gottesman *et al.*, 1990) whereas ClpX has only one (Gottesman *et al.*, 1993). In the presence of ATP and Mg^{2+} they form hexameric rings (Kessel *et al.*, 1995; Seol *et al.*, 1995; Grimaud *et al.*, 1998) that can act on their own as substrate-specific chaperones (Wickner *et al.*, 1994; Levchenko *et al.*, 1995; Wawrzynow *et al.*, 1995). ClpA or ClpX rings bind the ClpP core on either side of the chamber or on both to form a protease with specificity defined by the

ATPases (Kessel *et al.*, 1995). It has been shown *in vitro* that the ClpP core can be flanked by two different outer rings at the same time: ClpX on one side and ClpA on the other (Grimaud *et al.*, 1998). This architecture requires that the ATPase subunits act as gatekeepers, recognising the proper substrates and mediating their delivery to the proteolytic cavern (Hoskins *et al.*, 1998). A substrate molecule recognised by ClpA or ClpX is translocated through the chaperone cavity into the ClpP proteolytic chamber in an ATP-dependent manner. The products of degradation are short peptides of 5-10 amino acids that diffuse out after the reaction (Hoskins *et al.*, 1998). Protease specificity is defined by ATPase components although some substrates can be degraded by more than one protease. In the absence of peptidase components, the ATPases act as chaperones with the same substrate specificity as the appropriate proteases. ClpA, but not ClpX, activates the latent DNA-binding activity of the plasmid P1 replication initiator protein, RepA, by remodelling inactive dimers into active monomers (Wickner *at al.*, 1994; Pak & Wickner, 1997). In the presence of ClpP, ClpA tags RepA for the degradation by ClpAP (Wickner *et al.*, 1994; Hoskins *et al.*, 1998) as shown in figure 1.2. Similarly, ClpX disassembles MuA-DNA complexes and disaggregates λO; and ClpXP degrades MuA and λO (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993; Levchenko *et al.*, 1995; Wawrzynow *et al.*, 1995). In addition, ClpX chaperone participates in the initiation of replication of the RK2 plasmid by monomerising TrfA dimers, functional analogs of RepA (Konieczny & Helinski, 1997), and the ClpXP protease degrades the plasmid P1 addiction protein Phd (Lehnherr & Yarmolinsky, 1995) and the *E. coli* starvation sigma factor σ^S (Schweder *et al.*, 1996). ClpXP also regulates the formation of the UmuD′$_2$C DNA polymerase involved in SOS-mediated DNA repair (Frank *et al.*, 1996). Both ClpAP and ClpXP degrade defective proteins produced as the result of incomplete translation and therefore labelled by the SsrA-tagging system for degradation (Gottesman *et al.*, 1998).

ClpYQ. Like *clpPX*, the *clpQY* genes form an operon and their expression is induced by heat shock (Chuang *et al.*, 1993). ClpY is a ClpX-like ATPase with a single ATP-binding site (Missiakas *et al.*, 1996). It can form a ring structure that consists of either 6 or 7 subunits (Kessel *et al.*, 1996; Rohrwild *et al.*, 1997). ClpQ comprises two hexameric rings (Kessel *et al.*, 1996; Bochtler *et al.*, 1997; Rohrwild *et al.*,

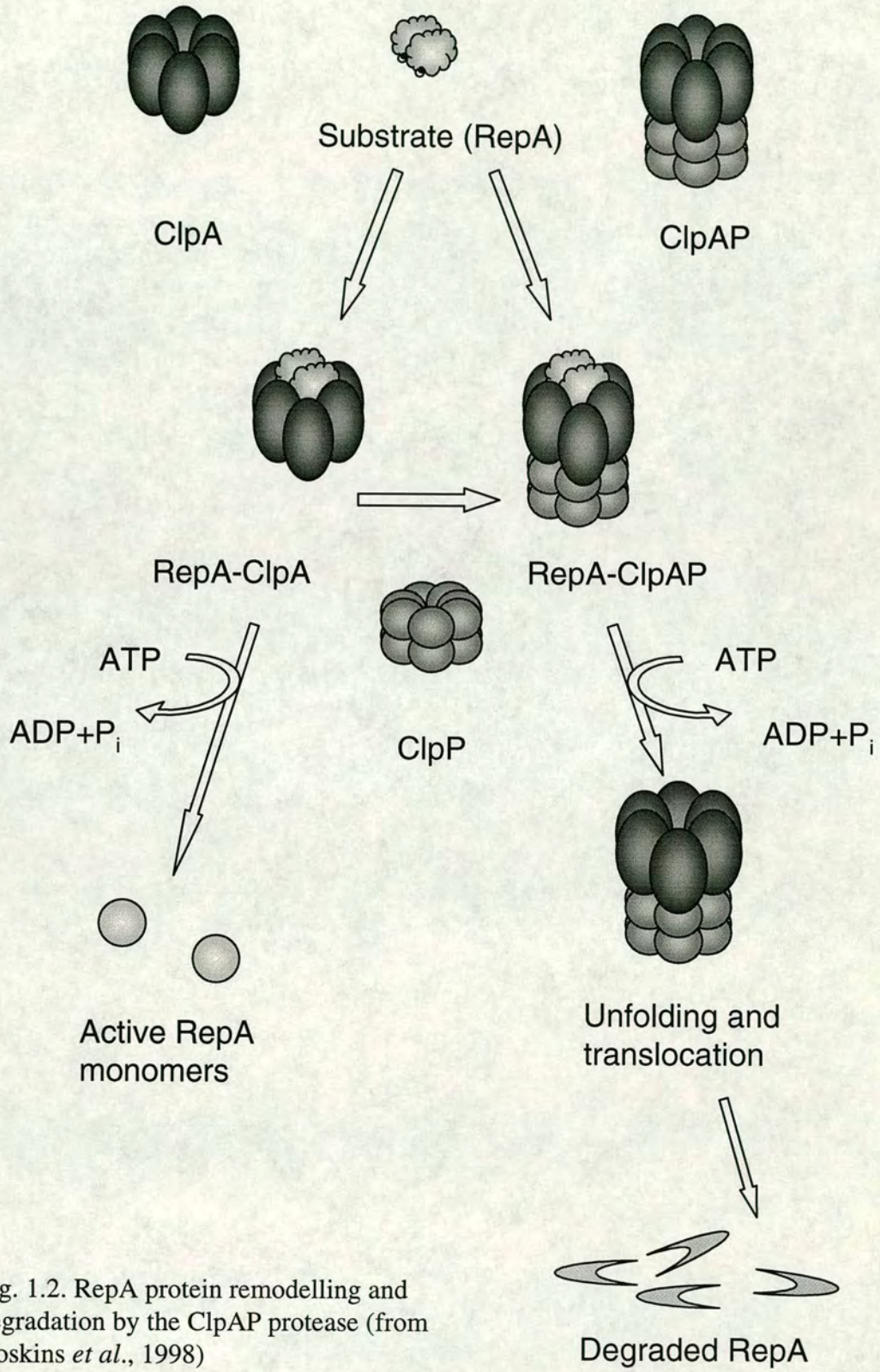


Fig. 1.2. RepA protein remodelling and degradation by the ClpAP protease (from Hoskins *et al.*, 1998)

1997). While ClpP is a serine peptidase, ClpQ degrades proteins by a threonine-dependent mechanism and resembles the 20S proteasome (Kessel *et al.*, 1996; Rohrwild *et al.*, 1996) found in all eukaryotic cells, as well as in archaebacteria and certain eubacteria (Dahlmann *et al.*, 1989; Tamura *et al.*, 1995). The three-dimensional structure of ClpYQ is similar to those of ClpAP and ClpXP; ring-shaped oligomers of ClpY and ClpQ surround a central cavity by forming a cylindrical particle with an overall composition of $Y_6Q_6Q_6Y_6$ or $Y_7Q_6Q_6Y_7$ (Kessel *et al.*, 1996; Rohrwild *et al.*, 1997).

SulA, an SOS-induced repressor of *E. coli* cell division, is one of two known natural substrates for ClpYQ (Seong *et al.*, 1999; Wu *et al.*, 1999). However, *clpQ* or *clpY* mutants do not suffer from SulA accumulation because SulA is degraded by another protease, Lon (Mizusawa & Gottesman, 1983). The effect of ClpYQ can be seen when *clpQY* are over-expressed in *lon*-deficient cells and suppress SOS-mediated inhibition of cell division because of ClpYQ-dependent degradation of SulA (Khattar, 1997; Seong *et al.*, 1999; Wu *et al.*, 1999).

The heat shock transcription factor σ^{32} is the other known substrate for ClpYQ (Kanemori *et al.*, 1997), which participates in its turnover along with the HflB protease (Herman *et al.*, 1995; Tomoyasu *et al.*, 1995).

ClpB. ClpB is another protein produced in response to heat treatment (Kitagawa *et al.*, 1991; Squires *et al.*, 1991). It shares homology with ClpA and resembles it in having two ATP-binding sites (Gottesman *et al.*, 1990; Kim *et al.*, 1998). It was believed that like ClpA, ClpB is a structural component of an ATP-dependent protease, however it does not interact with ClpP (Woo *et al.*, 1992) and no other peptidase partner for ClpB has been identified. ClpB is a tetramer that is an active ATPase on its own (Woo *et al.*, 1992). Possibly, ClpB acts only as a chaperone involved in refolding of heat-inactivated proteins (Laskowska *et al.*, 1996; Motohashi *et al.*, 1999).

Lon. The *lon* gene is closely linked to the *clpPX* operon and like the latter is σ^{32}-dependent (Goff *et al.*, 1984; Phillips *et al.*, 1984). The active Lon protease consists of four identical subunits of 87 kDa (Chin *et al.*, 1988; Goldberg *et al.*, 1994). Each subunit has a proteolytic domain with an active serine in the catalytic centre and an ATPase domain (Chin *et al.*, 1988; Fisher & Glockshuber, 1993). The overall

structure of the Lon protease is similar to those of the Clp proteases; it is a cylinder with a cavity inside but, unlike the Clp proteases, both ATPase and peptidase activities belong to the same monomer. The proteolytic domain can be expressed on its own and, like ClpP, it possesses peptidase activity (Rasulova *et al.*, 1998). If the protease activity of Lon is inactivated by a point mutation that leads to substitution of the active site serine, then the enzyme retains its ability to bind substrate, unfold it and move into the proteolytic chamber in an ATP-dependent manner. Over-expression of this mutant *lon* gene suppresses Lon-deficiency because even in the absence of degradation substrate molecules remain sequestered inside the proteases (Van Melderen & Gottesman, 1999).

Lon has been shown to play a primary role in the degradation of proteins unfolded and/or aggregated following heat treatment as well as unstable regulatory proteins. The latter include the λN protein (Gottesman *et al.*, 1981; Maurizi, 1987), the SulA cell division regulator (Mizusawa & Gottesman, 1983), the positive regulator of capsule synthesis RcsA (Torres-Cabassa & Gottesman, 1987), the F plasmid addiction system protein CcdA (Van Melderen *et al.*, 1994) and the UmuD component of the SOS-induced *E.coli* DNA polymerase V, $UmuD'_2C$ (Gonzalez *et al.*, 1998).

HflB. Among ATP-dependent proteases in *E.coli*, HflB is unique in that it is integrated into the cytoplasmic membrane by two N-terminal transmembrane segments (Tomoyasu *et al.*, 1993). The transmembrane region is required for homo-oligomerisation (Akiyama *et al.*, 1995), whereas the small periplasmic domain is important not only for homo-oligomerisation but also for association of HflB with the HflK-HflC membrane protein complex that modulates HflB activity (Kihara *et al.*, 1996, 1998; Akiyama *et al.*, 1998). The large cytosolic domain is responsible for ATP binding and Zn^{2+}-dependent proteolytic activity. Electron microscopy studies revealed ring-shaped structures formed by purified HflB (Shotland *et al.*, 1997). HflB degrades both cytosolically localised soluble proteins, such as σ^{32}, the IpxC deacetylase, the λ phage polypeptides CII and Xis (Herman *et al.*, 1995; Tomoyasu *et al.*, 1995; Shotland *et al.*, 1997; Leffers & Gottesman, 1998; Ogura *et al.*, 1999) and integral membrane ones, such as the SecY subunit of the protein translocase and YccA, whose function is unknown (Kihara *et al.*, 1995, 1998). There is evidence for

the involvement of HflB in the regulation of mRNA decay (Granger *et al.*, 1998; Wang *et al.*, 1998). HflB is essential, unlike other *E. coli* ATP-dependent proteases, because of its role in the regulation of the level of the IpxC deacetylase, an enzyme participating in the synthesis of membrane lipids (Ogura *et al.*, 1999).

1.3.2. Control of protein remodelling and degradation by regulating substrate recognition

Some proteins are constitutively degraded by specific proteases and in this case the level of a substrate protein in the cell will be dependent on the rate of its synthesis. SulA, a UV-inducible inhibitor of cell division, is degraded by both Lon and ClpYQ (Mizusawa & Gottesman, 1983; Seong *et al.*, 1999). During the SOS response the level of SulA is increased because of the derepression of *sulA* transcription; although the protein remains unstable. Shutting off the SOS response leads to repression of *sulA*, the residual amount of SulA is degraded and the cells can proceed with cell division. A similar constitutive proteolysis mechanism provides stability of some low copy number bacterial plasmids by selectively killing plasmid-free (cured) segregants or their progeny (Lehnherr & Yarmolinsky, 1995). The addiction module of plasmid prophage P1 consists of a pair of genes called *phd* and *doc*. Phd serves to prevent host death by neutralising cell toxin Doc. Constitutive degradation of Phd by ClpXP lowering its concentration in cells. Therefore, if a bacterium loses the plasmid, Phd will be diluted down quicker than Doc and the latter will kill the cell. A similar mechanism is described for the F plasmid where CcdB toxin is neutralised by the antidote CcdA that is constitutively degraded by Lon (Van Melderen *et al.*, 1994).

However, in many cases proteins are conditional substrates for proteolysis. They exist either as monomers or as components of complex structures that can be homo-polymers or include other proteins and DNA. In this case selective proteolysis occurs; a substrate is susceptible to degradation in one of the two or more states. λO protein is rapidly degraded by ClpXP (Wojtkowiak *et al.*, 1993) but it becomes protected from proteolysis in the pathway of the λ replication complex assembly when the λO-λP-DnaB preprimosome complex is formed (Wegrzyn *et al.*, 1995).

Both Lon and ClpXP are involved in the regulation of the assembly of the SOS-induced DNA polymerase UmuD′$_2$C. UmuD′ is a result of RecA-dependent autoproteolysis of UmuD (Shinagawa *et al.*, 1988; McDonald *et al.*, 1998). UmuD and UmuC are degraded by Lon (Frank *et al.*, 1996) and UmuD′ is a substrate for ClpXP, but only when it is paired with UmuD in the enzymatically inactive heterodimeric UmuD/UmuD′ complex (Frank *et al.*, 1996). Neither UmuD′/UmuD′ dimer nor the UmuD′$_2$C complex is recognised by the ClpXP protease. Such a mechanism allows cells to reduce the intracellular levels of the mutagenically active Umu proteins and thereby return to a resting state once error-prone DNA repair has occurred.

Control of the phage Mu replicative transposition is one of the well-understood examples of both chaperone- and proteolysis-dependent regulation provided by ClpXP. Mu transposase (MuA) monomers bind to specific sequences at each end of the phage genome and assemble a tetramer thereby pairing the two ends of the Mu DNA. The assembly and further steps of transposition are stimulated by MuB, another phage-encoded protein. The MuA tetramer cleaves the ends and joins the cleaved ends to a new DNA site by a reaction called strand transfer (Mizuuchi, 1992). Once the strand transfer is complete, the Mu DNA to be replicated requires removal of MuA and MuB. MuB dissociates on its own but the MuA-DNA complex is stable and has to be be disassembled by ClpX or ClpXP (Levchenko *et al.*, 1995; Kruklitis *et al.*, 1996). The presence of MuB protects MuA from premature attack by ClpX (Levchenko *et al.*, 1997a); its dissociation is a signal that the reaction of strand transfer is complete and MuA needs displacing. MuA can be either disassembled via ClpX chaperone activity or degraded by ClpXP (Levchenko *et al.*, 1995; Kruklitis *et al.*, 1996).

Investigation of the molecular mechanisms of substrate recognition by proteases sheds light on conditional susceptibility of substrates to remodelling and degradation. An understanding of the molecular determinants of substrate recognition by proteases requires two types of information: first, which features of the substrates are recognised and, second, how the proteases mediate this recognition. For many substrates it has been shown that either C-terminal or N-terminal sequence is important for the recognition. Mutations in these regions or their deletions stabilise

the substrates. The most critical test in identification of the target sequence recognised by proteases is the ability of this sequence to tag another, normally stable protein for proteolysis when it is fused to the latter. MuA is recognised by ClpX and the phage P22 repressor Arc is not. When the C-terminal sequence that includes the last 10 amino acids of MuA is fused to the C-end of Arc then the fusion protein is degraded by ClpXP (Levchenko *et al.*, 1997a). Another phage Mu protein, Mu repressor (mutated variant *vir*) is also degraded by ClpXP (Geuskens *et al.*, 1992; Mhammedi-Alaoui *et al.*, 1994) and 7 carboxyterminal amino acids were shown to make the protein susceptible to the protease. However, when the C-terminal sequence of the Mu repressor was fused to CcdA (a substrate for Lon) and CcdB, components of the F plasmid addiction module, only the former became a substrate for ClpXP while remaining a substrate for Lon (Laachouch *et al.*, 1996).

The C-terminus rule is also employed by the SsrA system to tag defective polypeptides for degradation. Proteins synthesised from mRNAs that lack a translational termination codon are modified by addition of a specific tail of 11 amino acids tail translated from the *ssrA* RNA (Keiler *et al.*, 1996). The role of this mechanism is both to terminate translation and release stalled ribosomes, and to tag incomplete proteins for degradation. The *ssrA* tail is recognised by ClpAP, ClpXP and HflB; in each case the proteases degrade the tagged proteins (Gottesman *et al.*, 1998; Herman *et al.*, 1998).

In contrast, the degradation of UmuD by Lon is dependent on internal sequences in a region of 24 N-terminal residues (Gonzalez *et al.*, 1998) and deletion of the first 18 amino acids of λO abolishes its ClpXP-dependent instability (Gonciarz-Swiatek *et al.*, 1999). An internal sequence of σ^S, remote from both termini, is important for proteolysis of σ^S by ClpXP (Schweder *et al.*, 1996).

It has been known for a few years that the regions involved in substrate recognition by the Clp proteases are located in ATPase subunits that define the substrate specificity. Proteins that are degraded by ClpXP are not recognised by ClpAP and vise versa (Wickner *et al.*, 1994). Later, it was shown that the C-terminal third of ClpX can bind proteins in a substrate-specific manner as efficiently as native ClpX (Levchenko *et al.*, 1997b). Recently, homologous substrate-recognition domains were found for Lon and Clp family proteases and chaperones and interactions of

these domains with target sequences of substrates were implicated in substrate discrimination by the chaperones and proteases (Smith *et al.*, 1999).

The mechanism of substrate recognition by proteases via interaction with a short amino acid sequence as a target allows regulation of proteolysis by making targets accessible or non-accessible for proteases. In the example of the ClpXP-dependent regulation of the phage Mu transposition, MuB protects MuA from attack by ClpX because MuB and ClpX interact with overlapping regions of MuA (Levchenko *et al.*, 1997a). As long as MuB is bound to MuA, the MuA target sequence remains unavailable for ClpX. A similar mechanism might operate the degradation of the λO protein, as well as the integral membrane protein SecY regulated by HflB. λO is stable as a component of the preprimosome complex λO-λP-DnaB bound to *ori*λ (Wegrzyn *et al.*, 1995; Zylicz *et al.*, 1998) but rapidly degraded by ClpXP as free protein (Wojtkowiak *et al.*, 1993), and SecY is stable when it is present in complexes with SecE, another subunit of the secretion system, but becomes unstable when produced in excess of SecE (Kihara *et al.*, 1995).

Stability of the starvation sigma factor, σ^S, depends on the physiological conditions of cells. The protein is unstable in exponentially growing cells but its half-life increases dramatically when cells enter stationary phase (Lange & Hengge-Aronis, 1994; Schweder *et al.*, 1996). The degradation has been shown to be ClpXP dependent (Schweder *et al.*, 1996). However, it requires another functional protein, RssB, which does not affect the stability of other known ClpXP substrates (Muffler *et al.*, 1996; Pratt & Silhavy, 1996). RssB is a stress response regulator that can exist in two forms: either phosphorylated or non-phosphorylated. Blocking phosphorylation abolishes ClpXP-dependent degradation of σ^S (Bouche *et al.*, 1998). When phosphorylated, RssB binds σ^S and this step is a prerequisite for proteolysis (Becker *et al.*, 1999). Therefore σ^S stability is regulated via RssB phosphorylation. In contrast to the examples of regulated proteolysis described above, for σ^S interaction of the substrate molecule with another protein does not protect it from proteolysis but is necessary to promote the degradation. Once bound to RssB, σ^S might undergo a conformational change, thereby, exposing the target and making it accessible for ClpXP.

It seems likely that most of the regulated proteolysis is dependent on substrate-specific protein interactions rather than on changes in protease availability or activity. There are, however, some exceptions to this. Bacteriophage T4 blocks proteolysis by Lon and other proteases; PinA, a T4 protein, inhibits the ATPase activity of Lon (Hilliard *et al.*, 1998a, 1998b). Similarly, bacteriophage lambda RexB protein inhibits ClpP (Engelberg-Kulka *et al.*, 1998).

For a long time the major role of proteolysis was thought to be one where proteases degrade unfolded and aggregated proteins. Intensive studies of proteolysis for the last decade have shown that it also plays an important regulatory role in life cycles of bacteria (for reviews see Gottesman, 1996, 1999). Regulation of protein synthesis and regulation of protein stability complement each other and allow bacteria to respond quickly to changing environmental conditions. Another important role of proteases or their chaperone components is to promote assembly or disassembly of multisubunit complexes. In the first case proteases can degrade one of the components and therefore regulate the ratio of the subunits involved in the complex formation. In the second, one or more subunits of the complex can be sequestered from the complex via ATP-dependent chaperone activity and either released or degraded. As more data accumulate, some other roles of proteases might be found. However, it is already clear that proteolysis is a very important part of the regulatory network operating cellular processes in bacteria.

CHAPTER 2. MATERIALS & METHODS

2.1. Bacteria

Bacterial strains used in this work are listed in table 2.1.

2.2. Bacteriophages

λ*vir* was used as a test phage for restriction and modification systems.

The *clpPX* alleles were cloned in the λ vector NM1151 (Murray, 1983). The λ*clpP*$^+$*X*$^+$ (λNM1357, constructed by A. Titheradge) includes a 6.2 kb *Bam*HI fragment from the Kohara phage 148 (Kohara *et al.*, 1987). A *clpX::kan* derivative (λNM1361) had the homologous *Bam*HI fragment from NK123, and a *clpP::cat* derivative (λNM1359) was made by inserting the *Hin*dIII-*Bam*HI fragment from pWPC16 (Maurizi *et al.*, 1990a). λNM1362, *clpP::cat clpX::kan* was made by excision of the prophage from the *clpP::cat clpX::kan* double mutant (SG22129) lysogenic for λNM1359.

λAD8, λAD10 and λTL25 used for assaying the p_{res} promoter are described in Loenen *et al.* (1987).

2.3. Plasmids

For the plasmids see table 2.2.

2.4. Standard solutions and buffers

Antibiotics:

- ampicillin: stock solution 100 mg/ml in dH_2O, 20-100 μg/ml final concentration.

-chloramphenicol: stock solution 10 mg/ml in 50% ethanol, 20 μg/ml final concentration.

-kanamycin: stock solution 10 mg/ml in dH_2O, 20 μg/ml final concentration.

-rifampicin: stock solution 10 mg/ml in methanol, 50 μg/ml final concentration.

Table 2.1. Bacterial strains

Strain	Relevant genotype or phenotype	Source or origin
E.coli K-12		
AB1157	$r_K^+m_K^+$ *rac-0*	De Witt & Adelberg (1962)
C600	$r_K^+m_K^+$	Appleyard (1954)
5K	C600 *hsdR514*	Hubacek & Glover (1970)
5KRI	$r_K^-m_K^+$ $r_{RI}^+m_{RI}^+$	N.E.Murray
CAG12148	*tsx-247*::Tn*10*	Singer *et al.* (1989)
CAG12017	*zba-3054*::Tn*10*	Singer *et al.* (1989)
CB51	*dam-3*	C.Boyd
DH5α	*endA1 hsdR17 recA1 gyrA* (Nal^r)	Woodcock *et al.* (1989)
DL1123	*mutH::kan*	D.R.L.Leach
ED8654	*hsdR514 supE44 supF58*	Murray *et al.* (1977)
HfrH	Hfr PO1	Low (1968)
JC3126	*ΔumuDC::cat*	D.R.L.Leach
JC9935	AB1157 *recA13 sup*°	D.R.L.Leach
KL16	Hfr PO45	Low (1968)
KL719	F^+ (F'101 $hsd_KR^+M^+S^+$)	Low (1968)
LE451	*rac-0 recA srl::*Tn*10*	Diaz *et al.* (1979)
NM181	$r_K^+m_K^+$ $r_{RI}^+m_{RI}^+$	N.E.Murray
NM477	C600 *ΔhsdMS*	Gough & Murray (1983)
NM510	*recBC*	N.E.Murray
NM654	C600 *ΔhsdRM*	Loenen *et al.* (1987)
NM659	*recA*::Cm	N.E.Murray
NM679	*Δ(hsdRMS)*	Webb *et al.* (1996)
NM765	*argA::*Tn*10 recD*	N.E.Murray
NM789	$\Delta hsdS_A$	Thorpe *et al.* (1997)
NM799	*hsdR*(A619V)	Webb *et al.* (1996)
NM840	C600 *gyrA96 ΔhsdRM* (r^-m^-)	NK31 × P1(NM654)
NM858	WA2899 *dnaC325 zjj*::Tn*10*	WA2899 × P1(TPC48)

NM863	WA2899 *hsdR*	NM858 × P1(WA2552)
MA156	*hflA150* Tn*10*	M. A. Hoyt
RH6972	*dnaQ::mini*Tn*10 (mutD)*	D.R.F.Leach
RS2	*topA10*	DiNardo *et al.* (1982)
SH6	*leu*::Tn*10*	K.J.Begg
TPC48	*dnaC325 zjj*::Tn*10*	Masters *et al.* (1989)
WA2552	$r_A^- m_A^+$ *(hsdR)*	Arber & Wauters-Willems (1970)
SG20252	MC4100 *lon-100 zba-3000*::Tn*10*	Trisler & Gottesman (1984)
SG21173	MC4100 *ΔclpA*::*kan*	Gottesman (1990)
SG22007	MC4100 *ΔclpP1*::*cat*	Maurizi *et al.* (1990)
SG22080	MC4100 *ΔclpX1*::*kan*	Gottesman *et al.* (1993)
SG22129	MC4100 *ΔclpP1*::*cat ΔclpX1*::*kan*	S. Gottesman
SG22192	MC4100 *ΔclpQ*::*cat*	W.-F.Wu & S.Gottesman
SG22193	MC4100 *ΔclpY*::*cat*	W.-F.Wu & S.Gottesman
WA2899	C600 $r_K^- m_K^-$ $r_A^+ m_A^+$	Fuller-Pace *et al.* (1985)
WA8304	*hflA150 hflB29 zgj25*::Tn*10*	Banuett *et al.* (1986)
XL1-Blue	*recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* ($r^- m^+$) *supE44 relA1 lac* [F'::Tn*10* (Tet^r) *proAB* $lacI^q \Delta(lacZ)M15$]	Bullock *et al.* (1987)
NK29	KL16 *recA*::Cm	KL16 × P1(NM659)
NK31	C600 *gyrA96*	C600 × P1(XL1-Blue)
NK113	AB1157 *ΔclpP1*::*cat*	AB1157 × P1(SG22007)
NK114	AB1157 *ΔclpX1*::*kan*	AB1157 × P1(SG22080)
NK115	C600 *gyrA96 ΔclpP1*::*cat*	NK31 × P1(SG22007)
NK116	C600 *gyrA96 ΔclpX1*::*kan*	NK31 × P1(SG22080)
NK121	NM840 *ΔclpP1*::*cat*	NM840 × P1(SG22007)
NK122	NK125 *ΔclpP1*::*cat*	NK125 × P1(SG22007)
NK123	NM840 *ΔclpX1*::*kan*	NM840 × P1(SG22080)
NK124	NK125 *ΔclpX1*::*kan*	NK125 × P1(SG22080)
NK125	NM840 *dnaC325 zjj*::Tn*10*	NM840 × P1(TPC48)

NK152	NM840 *lon-100 zba-3000*::Tn*10*	NM840 × P1(SG20252)
NK167	NM840 $hsd_AR^+M^+S^+$ *zjj*::Tn*10*	NK125 × P1(WA2899)
NK170	NM840 $hsd_AR^-M^+S^+$ *zjj*::Tn*10*	NK125 × P1(NM863)
NK180	NM840 *zba-3054*::Tn*10* $clpX^{hsdC}$	NM840 × P1(NK172)
NK188	NM840 *ΔclpA*::*kan*	NM840 × P1(SG21173)
NK190	NM840 *ΔclpQ*::*cat*	NM840 × P1(SG22192)
NK191	NM840 *ΔclpY*::*cat*	NM840 × P1(SG22193)
NK219	NM858 *ΔclpP1*::*cat*	NM858 × P1(SG22007)
NK220	NM858 *ΔclpX1*::*kan*	NM858 × P1(SG22080)
NK221	$hsd_AR^+M^+S^+$ *dnaC325 zjj*::Tn*10*	NM840 × P1(NM858)
NK228	NM840::Tn*10 hflA150*	NM840 × P1(MA156)
NK229	NM840 *hflB29 zgj25*::Tn*10*	NM840 × P1(WA8304)
NK231	NM477 *gyrA96 zjj*::Tn*10*	NM477 *gyrA* × P1(TPC48)
NK233	WA2899 *ΔclpP1*::*cat*	WA2899 × P1(SG22007)
NK234	WA2899 *ΔclpX1*::*kan*	WA2899 × P1(SG22080)
NK245	NK221 *Δhsd*	NK221 × P1(NM679)
NK246	NK245 *ΔclpX1*::*kan*	NK245 × P1(SG22080)
NK300	*rac-0* $recA^+srl^+$	LE451×P1(C600)
NK301	*rac-0 gyrA96*	NK300×P1(NK31)
NK302	*dam*	NK301×P1(CB51)
NK303	*ΔclpP1*::*cat*	NK301×P1(SG22007)
NK304	*ΔclpX1*::*kan*	NK301×P1(SG22080)
NK305	*argA*::Tn*10 recD*	NK301×P1(NM765)
NK306	arg^+ *recBC*	NK305×P1(NM510)
NK307	*ΔumuDC*::*cat*	NK301×P1(JC3126)
NK308	*recA*::Cm	NK301×P1(NM659)
NK309	*zjj*::Tn*10* $dnaC^{ts}$	NK301×P1(TPC48)
NK310	*hsdR*	NK301×P1(5K)
NK311	*Δ(hsdRMS)*	NK309×P1(NM679)
NK312	*Δ(hsdRMS) ΔclpX1*::*kan*	NK311×P1(SG22080)

NK313	*Δ(hsdRMS) recA::*Cm	NK311×P1(NM659)
NK315	*dam ΔclpX1::kan*	NK302×P1(SG22080)
NK320	*ΔclpX1::kan*	NK300×P1(SG22080)
NK323	*ΔclpX1::kan recA::*Cm	NK304×P1(NM659)
NK324	*Δ(hsdRMS) ΔclpX1::kan recA::*Cm	NK312×P1(NM659)
NK325	*hsdR ΔclpX1::kan*	NK310×P1(SG22080)
NK326	*mutD*	NK301×P1(RH6972)
NK327	*mutD ΔclpX1::kan*	NK326×P1(SG22080)
NK329	*topA10 ΔclpP1::cat ΔclpX1::kan*	RS2×P1(SG22129)
NK331	*mutH::kan*	NK301×P1(DL1123)
NK351	*hsdR*(A619V)	NK309×P1(NM799)
NK352	*Δ(hsdMS)5*	NK309×P1(NM477)
NK354	hsd_K^- hsd_A^+	NK309×P1(NK167)
NK355	hsd_K^- hsd_A^+ *ΔclpX1::kan*	NK354×P1(SG22080)
<u>*E.coli* C</u>		
JR300	wild type r^0m^0	Prakash-Cheng *et al.* (1993)
JR302	JR300 *recA* Kan^r *hsdC* ($clpX^{hsdC}$)	Prakash-Cheng *et al.* (1993)
NK38	JR300 *gyrA96*	JR300 × P1(XL1-Blue)
NM820	JR302 $recA^+$	O'Neill *et al.* (1997)
NK84	*leu*::Tn*10 lac*	NM820 × P1(SH6); Amp-enrichment to obtain the *lac* derivative
NK85	NK84 leu^+	NK84 × P1(HfrH)
NK105	*gyrA96 recA*::Cm HsdC-like	NK29 × NK38
NK107	NK105 $recA^+$	NK105 × P1(HfrH)
NK172	NM820 *zba-3054*::Tn*10*	NM820 × P1(CAG12017)
NK176	NK38 *zba-3054*::Tn*10* $clpX^{hsdC}$	NK38 × P1(NK172)

Table 2.2. Plasmids

Plasmid	Description	Source or reference
F′101	hsd_K^+	Low (1972)
F′101-101	hsd_K^+ *zjj*::Tn*10*	Prakash-Cheng *et al.* (1993)
F′101-102	$hsd_K R^- M^+ S^+$ *zjj*::Tn*10*	Prakash-Cheng *et al.* (1993)
F′101-201	hsd_K^+ *zjj*::Tn*10* miniTn*5*-Cm	F′101-101::miniTn*5*-Cm
F′101-202	$hsd_K R^- M^+ S^+$ *zjj*::Tn*10* miniTn*5*-Cm	F′101-102::miniTn*5*-Cm
F′101-301	hsd_A^+ *zjj*::Tn*10* miniTn*5*-Cm	See 3.3.1
F′101-302	$hsd_A R^- M^+ S^+$ *zjj*::Tn*10* miniTn*5*-Cm	See 3.3.1
pACYC184	p15A replicon, Cm^r Tet^r	Chang & Cohen (1978)
pBCBH1	pUC18 $hsd_K M^+$	O'Neill *et al.* (1998)
pBE3	pBR322 hsd_K^+ Amp^r Tet^s	O'Neill *et al.* (1997)
pBE3*	pBR322 $hsd_K R^- M^+ S^+$ Amp^r Tet^s	O'Neill *et al.* (1997)
pBg3	pBR322 $hsd_K R^+ M^+$ Amp^r Tet^s	Loenen *et al.* (1987)
pBR322	pMB1 replicon, Amp^r Tet^r	Bolivar *et al.* (1977)
pBRK	pBR322 Amp^s Tet^r Km^r	*kan* is inserted in the *Pst*I site of pBR322.
pFFP30	pBR322 hsd_A^+ Amp^r Tet^s	Fuller-Pace *et al.* (1985)
pFFP31	pBR322 $hsd_A M^+ S^+$ Amp^r Tet^s	Fuller-Pace *et al.* (1985)
pJES23	pJF118 $hsd_K M^+ S^+$ Amp^r	O'Neill *et al.* (1998)
pJH16	$r_{RI}^- m_{RI}^+$ Cm^r	Heitman *et al.* (1989)
pJK2	pBR322 $hsd_K R^+$ Amp^r Tet^s	Kelleher *et al.* (1991)
pNK3	pACYC184 $hsd_K R^+$ Cm^r Tet^s	*Hin*dIII-*Sma*I fragment of pBg3 ligated in pACYC184 digested with *Hin*dIII and *Nru*I.
pNK6	pUC18 $hsd_K S^+$	pUES6 Δ*Age*I
pUES6	pUC18 $hsd_K M^+ S^+$	O'Neill *et al.* (1998)

-streptomycin: stock solution 10 mg/ml in dH_2O, 20 µg/ml final concentration.

-tetracycline: stock solution 10 mg/ml in 50% ethanol, 10 µg/ml final concentration.

Antibiotic solutions were stored in the dark at 4°C except ampicillin which was stored at –20°C.

Ethidium bromide: 10 mg/ml in dH_2O, stored in the dark at 4°C.

Tris-EDTA (TE): 10 mM Tris-HCl, 1 mM EDTA, pH 8.

Tris-Borate-EDTA (TBE) 20×: 1.78 M Tris base, 1.78 M boric acid and 0.05 M EDTA.

Tris-buffered saline (TBS): 10 mM Tris-HCl, 150 mM NaCl, pH 7.5.

SSC, 20×: 175.3 g of NaCl and 88.2 g of sodium citrate dissolved in dH_2O, adjusted pH to 7.0 with NaOH and the volume to 1 l with dH_2O.

Phage buffer: KH_2PO_4 (3 g), Na_2PO_4 (7 g), NaCl (5 g), $MgSO_4 \cdot H_2O$ (1 ml of 0.1 M), $CaCl_2$ (10 ml of 0.1 M), gelatine (1 ml, 1% w/v), and H_2O to 1 l.

MC-buffer: 0.1 M $MgSO_4$, 5 mM $CaCl_2$.

Amino acids, 100×: 2 mg/ml of L-forms and 4 mg/ml of DL-forms, dissolved in dH_2O and autoclaved.

Thiamine, 100×: 1 mg/ml, dissolved in dH_2O and autoclaved.

Sugars (lactose, glucose and others), 100×: 20%, dissolved in dH_2O and autoclaved.

2-aminopurine, 100×: 40 mg/ml, dissolved in dH_2O and stored at 4°C.

MMS, 50×: 1% (v/v) aqueous solution, stored at -20°C

2.5. Media

Spizizen minimal salt (5×): $(NH_4)_2SO_4$ (10 g), K_2HPO_4 (70 g), KH_2PO_4 (30 g), tri-Na citrate (5 g) and $MgSO_4$ (1 g) were dissolved in dH_2O to a final volume of 1 l and autoclaved.

Luria (L) Broth: Difco Bacto yeast extract (5 g/l), NaCl (10 g/l), pH 7.2.

Minimal Broth: 5× Spizizen minimal salt solution (80 ml) was diluted with 320 ml sterile dH_2O and appropriate sugar, amino acids and vitamins were added.

Luria broth and minimal broth were converted to solid media by adding 15 g/l Difco agar.

Baltimore Biological Laboratory (BBL) Agar: trypticase (10 g/l). NaCl (5 g/l), Difco agar (10 g/l; top layer 6.5 g/l).

Eosin-Methylene Blue (EMB) Agar: L-agar supplemented with 0.04% eosin yellow, 0.0065% methylene blue and 0.5% sugar. 100× stock solutions of eosin yellow (4%) and methylene blue (0.65%) were prepared (autoclaved) and added to molten L-agar along with the sugar before pouring plates.

All media were sterilised by autoclaving at 15 lb in^{-2} for 15 min.

2.6. Microbiological and genetic techniques

2.6.1. Long-term storage of bacterial cells

A single freshly grown colony was picked with a loop and stabbed into a thick layer of L-agar in a small bottle. The stabs were incubated overnight at 30 or 37°C to allow bacterial growth and kept at room temperature.

2.6.2. Preparation of plating cells

An overnight culture was diluted 50-fold in L-broth with 1% maltose, grown to mid-log phase, and the cells were harvested by centrifugation (3,000 × g, 5 min) and resuspended in the same volume of 10 mM $MgSO_4$.

2.6.3. Preparation of λ lysates

A single plaque was picked using a toothpick and resuspended in 1 ml phage buffer containing a few drops of chloroform (~10^7 p.f.u./ml). 0.1 ml of an appropriate dilution of the phage, to give confluent lysis (~10^6 p.f.u.), was mixed with an equal volume of plating cells, left for 15 min to allow phage adsorption, and 2.5 ml molten BBL top agar was added before the mixture was poured on fresh BBL-agar in a plate. The plate was incubated until confluent lysis was observed (6-7h) and placed at 4°C overnight with an overlay of L-broth (4 ml). The broth was decanted, remaining cells lysed with a few drops of chloroform, and clarified by centrifugation (3,000 × g, 5 min). The phage lysate was transferred to a fresh bottle and stored at 4°C.

2.6.4. Restriction assays

Bacteria to be tested for restriction and a control restriction-deficient strain were grown until mid-log phase and 1 ml of the cultures were mixed with 0.1 ml aliquots of the appropriate dilution of λ.0 lysate. After 15 min incubation at room temperature to allow phage adsorbtion, 2.5 ml BBL-top agar was added and used to overlay a BBL-bottom agar plate. Plaques were counted after overnight incubation and the ratio of p.f.u. on the tested strain to that one on the control was taken as an e.o.p. The reverse ratio displayed restriction. The experiments were accompanied by a control of λ.K or λ.A.

For a quick non-quantitative test, 10 µl of appropriate dilutions of λ.0 and λ.K (or λ.A) were spotted on lawns of test-bacteria and two control strains (restriction-proficient and restriction-deficient). After overnight incubation λ.0 produced a spot of confluent lysis on restriction-deficient bacteria and a few plaques on the other control strain. Test bacteria were compared with the controls.

2.6.5. Modification assays

A λ plaque grown on a strain to assay for modification was picked with a toothpick, resuspended in 1 ml phage buffer, treated with a drop of chloroform and the titre of the phage was determined on restriction-proficient and restriction deficient bacteria. If the phages are modified they form plaques with the same efficiency on both strains.

2.6.6. Single round of infection of the phage λ

1 ml of bacterial culture grown in L-broth till mid-log phase was mixed in an Eppendorf tube with 10 µl $MgSO_4$ (1M) and 10^7 phages. The mixture was incubated at 37°C with aeration using a rotating wheel. After 1h, 0.1 ml of the sample was diluted in 0.9 ml phage buffer and treated with 10 µl chloroform for 30 sec to kill bacteria. The phage progeny were analysed for modification as described above.

2.6.7. Lysogenisation of *E. coli* with λ and λ/*E. coli* chromosome allele exchange

The phage used contained a temperature sensitive mutation (*c*I857) that prevents the maintenance of the lysogenisation state at temperatures above 37°C and a mutation in

their attachment site. The latter mutation forces integration of the phage into the bacterial chromosome via homologous recombination between the chromosome and a fragment cloned in the phage. The lysate was diluted 100-fold in phage buffer and spotted (10 µl) onto a lawn of bacteria. The spots were dried and the plate was incubated overnight at 32°C to allow lysogenisation. Bacteria from the spots were streaked with a toothpick on L-agar seeded with λ*imm*^{21}*c*Ι and h^{82}*imm*^{21}*c*Ι, homoimmune phages (the λNM1151 vector is *imm*^{21}) which lyse cells that do not contain a prophage and therefore select for lysogens. Alternatively, when the phage, used for lysogenisation had a drug resistance gene, bacteria from the spots were streaked on agar with an appropriate antibiotic. The plates were incubated overnight at 32°C and single colonies were purified at the same temperature and tested for the presence of a prophage (lysogens survive infection by homoimmune phages but are killed by λ*vir*).

The prophage can be induced at 42°C. On excision via homologous recombination, allelic exchange can occur between the sequences common to the λ and the bacterial chromosome. The phage progeny can be analysed for allele exchange and phage with a new genotype can be obtained *in vivo*. In my experiments λ*clpP::cat clpX*$^{+}$ was used to lysogenise *clpP::cat clpX::kan* bacteria and after the prophage were induced the progeny were screened for their ability to produce Km^{r} lysogens on Km^{s} bacteria to isolate λ*clpP::cat clpX::kan*.

2.6.8. Conjugation

Cultures of donor and recipient bacteria grown in LB-broth till middle log-phase were mixed in a large tube. The ratio of donor : recipient was dependent on the purpose of any particular experiment. It was 1:10 for the mapping experiments, 10:1 to test the effect of establishment of a new specificity on survival of recipient bacteria and 1:1 for all other cases. Cross mixtures were incubated with gentle aeration on a rotating wheel for 2-3 hours, and appropriate dilutions were plated on selective media.

2.6.9. P1 transduction

Preparation of P1 lysates. Over-night cultures were diluted 50-fold in L-broth with 5mM $CaCl_2$ and incubated till late log-phase. 1ml samples were mixed with 10^5- 10^6 P1 phages in small test tubes and incubated in a 37°C water bath for 15-20 min. 2.5 ml of BBL-top agar was added to each tube, mixed and poured in a freshly-prepared plate with BBL-bottom agar supplemented with 5mM $CaCl_2$. Plates were incubated at 37°C for 6-7 hours or overnight. Confluent lysis of bacteria was observed. 2-3 ml of L-broth was added per plate and the top layers were removed, treated for 30 seconds with chloroform and vortexed. After centrifugation (3,000 × g, 5 min) the supernatants containing P1 were removed, titred and kept at 4°C.

P1 transduction. Bacteria grown till stationary phase in L-broth were harvested by centrifugation and resuspended in MC-buffer in 1/10 of the original volume. 0.1 ml of cells was mixed with 0.1 ml of P1 lysate in one cross and with 0.01 ml in the other and the cross mixtures were incubated in a 37°C water bath for 15-20 min. Sodium citrate was added to a final concentration of 100 mM and the mixtures were plated on selective agar media. After over-night incubation at the appropriate temperature the colonies were purified and used for further experiments.

2.6.10. Transformation

Electroporation and Ca^{2+}-treatment were used to make cells competent in DNA uptake.

Electroporation. Overnight cultures of bacteria were diluted 100-fold in flasks with L-broth and incubated at 37°C with vigorous aeration. When OD_{600} reached 0.5-0.6 the cultures were chilled by putting the flasks in an ice-water bath for 5 min. The bacteria were transferred into pre-chilled McCartney bottles, harvested by centrifugation at 4°C and washed 3 times in ice-cold dH_2O. The fourth wash was done in 10% ice-cold glycerol and the cell pellets were resuspended in 1/1000 of the initial volume of cultures grown in broth. 20 µl aliquots were transferred in pre-chilled eppendorf tubes, frozen on dry ice with ethanol and stored at –70°C. For electroporation the aliquots were thawed on ice and 1-2 µl of DNA containing as little salt as possible was added. The mixtures were transferred to electroporation cuvettes (0.1cm) pre-chilled on ice and pulsed with an electric field using a Gene

Pulser (Bio-Rad) electroporator (settings, 25 μF, 1.7 kV, 400 Ω). LB-broth supplemented with 0.4% glucose (5 ml) was added immediately to the mixture transferred to a 10 ml bottle and incubated for 1 h at 37°C with aeration. Samples were plated on selective agar and incubated overnight.

Ca^{2+}-method. Overnight cultures of bacteria were diluted 50-fold in flasks with LB-broth and incubated at 37°C with vigorous aeration. When OD_{600} reached 0.4-0.6 the cultures were chilled by putting the flasks in an ice-water bath for 5 min. The bacteria were transferred into pre-chilled McCartney bottles, harvested by centrifugation at 4°C, resuspended in ice-cold 0.1M $CaCl_2$ (1/2 of the initial volume) and incubated on ice for 1 hour. The cells were centrifuged again and resuspended in ice-cold 0.1M $CaCl_2$ and 20% glycerol (1/20 of the initial volume). 0.1 ml aliquots were transferred to ice-cold eppendorf tubes and stored at –70°C. For transformation the aliquots were thawed on ice, mixed with up to 10 μl of DNA solution and the mixtures were incubated on ice for at least 30 min. The tubes were transferred to 42°C (water bath) for two minutes and returned to ice for one minute. 1 ml of L-broth was added in each tube and the tubes were incubated at 37°C for 1h. Bacteria were plated on selective media and incubated overnight to obtain transformants.

2.6.11. Ampicillin enrichment

Ampicillin enrichment for Lac^- phenotype followed by plating on EMB-lactose indicative agar was used to select for spontaneous *lac* mutations. Bacteria were grown in minimal media supplemented with glucose till early log-phase, washed twice and resuspended in minimal media with lactose. After an hour of growth in lactose medium, ampicillin was added to a final concentration of 100 μg/ml and the incubation was continued for the next two hours. Cells were washed to remove ampicillin, diluted in L-broth and plated on EMB-lactose agar. Light colonies from EMB-lactose plates were purified and tested for their ability to use lactose, glucose and galactose as the sole source of carbohydrate in minimal medium. An isolate that did not grow on lactose but grew on the other carbohydrates (NK84) was used for further work.

2.6.12. Construction of miniTn*5*-Cm derivatives of F′ plasmids

MiniTn*5*-Cm confers a resistance to chloramphenicol that can be used as a marker to select for transconjugants. pUT/miniTn*5*-Cm (Amp^r Cm^r) is a *tra*$^-$ plasmid with the *mob*-site of RP4, a conjugative plasmid (de Lorenzo *et al.*, 1990). The *tra* region of RP4 is inserted in the chromosome of the host strain, therefore, pUT/miniTn*5*-Cm can be transferred from the host strain to other bacteria. The host strain is a lysogen for λ*pir*; the pir gene encodes π-protein. The plasmid requires π-protein for its replication and therefore it is unstable in most *E. coli* strains because they do not produce the protein. Therefore, transfer of the plasmid into another *E. coli* strain, followed by selection and screening for Cm^rAmp^s colonies, leads to survival of bacteria with insertions of the miniTn*5*-Cm transposon into their chromosomes or plasmids. If the transposon is on a conjugative plasmid, such as F, then Cm^r can be transferred to a recipient with high efficiency detectable in replica plating matings.

BW (pUT/miniTn5-Cm) was conjugated with CSH50 (F′101-101 or F′101-102) and Str^r Tet^r Cm^r transconjugants were selected and screened for Amp^s and ability to transfer Cm^r in replica plating matings with a Nal^r recipient. The resultant plasmids, named F′101-201 and F′101-202, confer both Tet^r and Cm^r.

2.6.13. Restriction alleviation

2-AP (400 μg/ml) was added to mid-log cultures grown at 37°C in L-broth. Intensive aeration was provided before and during the treatment. After 1h, the cells were washed, resuspended in fresh broth and tested for restriction. UV induced RA was measured as described in Kelleher & Raleigh (1994), RA in response to nalidixic acid as described by Thoms & Wackernagel (1984).

2.7. Manipulation of nucleic acids

2.7.1. Small-scale preparation of plasmid DNA

Bacteria were grown till late log phase or overnight in L-broth with an appropriate antibiotic to maintain plasmids in the population. 5 ml of culture was applied to plasmid DNA purification procedure using QIAprep Spin Miniprep Kit (Qiagen).

2.7.2. Small-scale preparation of phage λ DNA

A culture of an appropriate host was diluted 40-fold in L-broth (4 ml), supplemented with $MgSO_4$ (10 mM), infected with the required phage (~10^8 pfu) and grown with aeration until lysis (normally, 37°C for 4 h). Remaining cells were lysed by the addition of chloroform (0.1 ml), cell debris was removed by centrifugation (3,000 × g, 10 min), and nucleases (5 µl, 10 mg/ml RNase and DNase) added to supernatant in a fresh tube. The lysate was incubated at 37°C for 30 min, and the phage precipitated by the addition of polyethylene glycol (PEG) and salt (4 ml; 20 g 6K PEG, 11.7 g NaCl, 78 ml phage buffer). The mixture was left overnight at 4°C, and the phage particles sedimented by centrifugation (13,000 × g, 20 min, 4°C). Care was taken to remove most of the PEG and the phages were resuspended in phage buffer (0.5 ml). Chloroform was added (0.5 ml) to remove any remaining PEG, the mixture clarified by centrifugation (5,000 × g, 3 min) and the aqueous phase containing the phage particles was transferred to a tube containing phenol (0.5 ml) and TE buffer (0.1 ml). The two phases were separated by centrifugation (11,000 × g, 3 min), and the aqueous phase transferred to a tube containing phenol (0.25 ml) and chloroform (0.25 ml). The aqueous phase containing naked phage DNA was separated as before, mixed with an equal volume of chloroform and the aqueous phase collected. To remove some of the excess salt carried over from the stages with phage particles, the DNA in solution was precipitated with ethanol twice. 1/10 volume of sodium acetate (3 M, pH 5.3) and two volumes of ethanol were added and the solution was left at –20°C for 20 min. The DNA was sedimented by centrifugation (11,000 × g, 20 min), rinsed with 70 % ethanol, dried, resuspended in TE (0.1 ml) and after the ethanol precipitation was repeated the DNA was stored at –20°C.

2.7.3. Small-scale preparation of chromosomal DNA

Chromosomal DNA was purified from 5 ml overnight culture using Genomic DNA Purification Kit (Edge BioSystems) according to the manufacture's recommendations.

2.7.4. Agarose gel electrophoresis

Agarose gels for electrophoretic analysis of DNA were prepared with TBE buffer. Agarose concentration varied from 0.3 to 1% according to the sizes of expected DNA fragments. Samples containing 1× loading dye (6 × stock is 0.25% bromophenol blue, 0.25% xylene cyanol and 40% (w/v) sucrose in H_2O) were always loaded after immersion of the gels in TBE buffer with 0.5 μg/ml of ethidium bromide. The gels were run at 50-70 mA at room temperature. These conditions usually led to good fragment separation in less than an hour. For better separation, especially with larger fragments and higher agarose concentration, the gels were run at 20-40 mA for several hours. After the run the DNA was visualised by using UV transilluminator.

2.7.5. Cutting of DNA with type II restriction enzymes

Endonuclease reactions were performed in a volume of 10-50 μl containing up to 1-2 μg of DNA. The reactions contained the appropriate Boehringer Mannheim or New England Biolabs restriction buffer at 1 × concentration. BSA was added (to 1 mg/ml) if recommended by the manufacturer of an enzyme. The digests were made up to their final volume using dH_2O. The complete restriction mixtures were incubated at the temperature recommended for a particular enzyme for 1–4 h. The products of the reaction were analysed using agarose gel electrophoresis. If digested DNA was used for ligation the restriction enzymes were heat inactivated (20 min at 65°C or 80°C according to supplier's recommendations). When an enzyme could not be heat inactivated, an AG cartridge (Advanced Genetic Technologies Corp.) that allows quick DNA purification from any protein was used. If the DNA was to be subjected to purification from an agarose gel, it was not necessary to inactivate the enzyme.

2.7.6. Recovery of DNA from agarose gels

DNA was purified from agarose gel slices using a DNA Purification Kit II (Hybaid).

2.7.7. Ligation of DNA

DNA ligation was usually performed in a final volume of 10-20 μl. The reactions contained between 0.5–2 μg of total DNA with insert DNA in a 2- to 5-fold molar

excess over the vector DNA, 1 × New England Biolabs ligation buffer and T4 DNA ligase. The reactions were incubated overnight at 16°C. 5-10 µl of the reaction mixture was directly used to transform competent cells of an appropriate strain of *E. coli*. When λ vectors were used the ligase was heat inactivated and the ligated DNA was packaged using λ packaging extracts (see below).

2.7.8. Phage λ DNA packaging

A 50 µl aliquot of λ packaging extract (Promega) stored at –70°C was defrosted on ice. 15 µl of the extract was mixed with 10 µl of ligation reaction with inactivated ligase and containing at least 200 ng of DNA. The mix was left at room temperature for 2 h. 0.5 ml of phage buffer was added and appropriate dilutions were plated on *E. coli* ED8654. After overnight incubation the plaques were analysed by Southern blotting. Alternatively, when the cloned fragment contained a drug resistance marker, phages from single plaques were respotted on a lawn of bacteria. Survivals from the spots were streaked on agar with the appropriate antibiotic to screen for drug-resistant lysogens. DNA from selected λ clones was purified and analysed by restriction digest.

2.7.9. Southern blotting

Transfer of DNA from plaques to nylon membranes. Cells, preadsorbed with phage (3-5 × 10^3 pfu/ml) were plated in BBL-top agar containing $MgSO_4$ (10mM) on dry BBL plates. After overnight incubation at 37°C, the plates were placed at 4°C for 2 h to harden the agar. After this time, dry nylon filter discs were placed on the surface of the plates for 2 min to allow transfer of the phage and DNA. The filters were peeled from the plates and placed, DNA side up, onto a pad of blotting paper soaked with NaOH (0.5 M) for 5 min. The filters were next immersed in denaturation buffer (0.1 M NaOH, 1.5 M NaCl) for 20 sec and then dipped twice in neutralisation buffer (0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5) and once in 2 × SSC for 20 sec each. The filters were briefly blotted and DNA was cross-linked by UV irradiation in a Stratagene Stratalinker (12,000 joules/cm^2).

Transfer of DNA from agarose gels to nylon membranes. The agarose gel containing DNA to be analysed was placed in 0.25 M HCl for 15 min. After rinsing in dH_2O (10

min) the gel was immersed in denaturing solution (0.5 M NaOH, 1,5 M NaCl) for 30 min. The gel was rinsed again in dH_2O (10 min) and then neutralised in ammonium acetate (1 M, pH 8, 1h). For transfer, the gel was placed on a glass plate. A nylon membrane, cut to the same dimensions as the gel, was briefly soaked in ammonium acetate and placed on top of the gel. Three pieces of blotting paper of the same size as the gel were wetted in ammonium acetate and placed on top of the membrane. The blotting paper sheets were covered by a 1 cm stack of dry paper towels. A glass plate, weighed down by a beaker of water, ensured even transfer. Transfer was continued overnight. The membrane was rinsed briefly in 2 × SSC and the DNA was cross-linked to the membrane using a Stratagene Stratalinker (12,000 joules/cm^2). The gel was restained with ethidium bromide to verify that the DNA had been transferred.

Labelling of DNA probe and hybridisation. The Boehringer Mannheim non-radioactive labelling and detection kit was used to detect specific DNA sequences in Southern hybridisation. The kit is based on the incorporation of dNTPs coupled to digoxigenin during random-primed DNA synthesis by Klenow enzyme using a ssDNA template. The reaction mixture included DNA template (0.5-150 µg/ml), hexanucleotide mixture (1×), dNTP labelling mixture (1×) and Klenow enzyme (100 units/ml) in a final volume of 20 µl. The reaction was incubated for 1 h at 37°C and the reaction was stopped by heat inactivation of the enzyme (75°C, 20 min).

The membranes with cross-linked DNA were sealed in a small plastic bag with hybridisation buffer (no bubbles) and incubated for 1 h at 37°C with gentle shaking (prehybridisation step). The probe was diluted in 0.1 ml of hybridisation buffer, boiled for 10 min to denature the DNA and after a short spin in a microcentrifuge cooled on ice and dissolved in an appropriate volume of hybridisation buffer solution (the final concentration of the probe was ~15 ng/ml). The bag was unsealed and the buffer was poured out and replaced with the probe. The bag was sealed again and incubated overnight at 37°C (hybridisation step). Next day the membranes were washed 2 × 5 min in 2 × SSC, 0.1% SDS and then 2 × 15 min in 1 × SSC, 0.1% SDS. The membranes were incubated in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20) for 5 min, then in blocking solution (1% block in the washing buffer) for 30 min and in blocking solution with the anti-digoxigenin antibody

conjugated to alkaline phosphatase (1:10000) for another 30 min. The membranes were washed with washing buffer 2 × 15 min and incubated for 5 min in 0.1 M Tris-HCl, 0.1 M NaCl, 25 mM $MgCl_2$ (pH 9.5). The membranes were transferred onto Saranwrap film (after allowing the liquid to drip off the membranes) and spotted with a chemiluminescent substrate for alkaline phosphatase (SDP-Star) diluted 1:400 in 0.1 M Tris-HCl, 0.1 M NaCl, 25 mM $MgCl_2$ (pH 9.5). As the dephosphorylated substrate decomposes via an unstable intermediate, light is emitted which may be captured on X-ray film.

2.8. Manipulation of proteins

2.8.1. Tris-glycine SDS page

Solutions:

- 4 × stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

  15.25 g of Tris base was dissolved in 200 ml dH_2O, adjusted to pH 6.8 with concentrated HCl, made up to 250 ml, filtered and kept at 4°C.

- 4 × resolving gel buffer (1.5 M Tris-HCl, pH 8.8)

  45.5 g of Tris base was dissolved in 200 ml dH_2O, adjusted to pH 8.8 with concentrated HCl, made up to 250 ml, filtered and kept at 4°C.

- 10% SDS (w/v)

  20 g of SDS was dissolved in 150 ml dH_2O, made up to 200 ml, filtered and kept at room temperature.

- 10% ammonium persulphate (w/v) – freshly made

  0.2 g of ammonium persulphate was dissolved in 2 ml dH_2O.

- reservour buffer

  3 g of Tris base and 144 g of glycine were dissolved in 990 ml dH_2O. SDS was added to final concentration 0.1% (10 ml of 10% stock solution).

- 2 × loading buffer

  4 × stacking gel buffer (2.5 ml), 10% SDS (2 ml), glycerol (2 ml), dH_2O (2.5 ml) β-mercaptoethanol (1 ml), bromophenol blue (some crystals to give blue color).

E. coli proteins were routinely separated using SDS–polyacrylamide gel electrophoresis with a discontinuous buffer system (Laemmli, 1970). Usually a 7.5% resolving gel and a 5% stacking gel were employed and a mini–gel apparatus (SE 250) manufactured by Hoeffer Scientific Instruments was used. The eperiments on HsdR stability required better resolution of high molecular weight proteins and therefore larger gels (14×14 cm) and lower percentage acrylamide (6% for the resolving gel and 3% for the stacking gel) were used. The composition of typical gel solutions was as follows:

Stock solutions	Resolving gel		Stacking gel	
	6%	7.5%	3%	5%
- acrylamide stock solution (30%)	4 ml	5 ml	1 ml	1.7 ml
- 4 × resolving gel buffer	5 ml	5 ml	-	-
- 4 × stacking gel buffer	-	-	2.5 ml	2.5 ml
- SDS (10%)	0.2 ml	0.2 ml	0.1 ml	0.1 ml
- dH_2O	10.6 ml	9.6 ml	6.3 ml	5.6 ml
- ammonium persulphate (10%)	0.2 ml	0.2 ml	0.15 ml	0.15 ml
- TEMED	0.02 ml	0.02 ml	0.015ml	0.015 ml
Total volume	20 ml	20 ml	10 ml	10 ml

These solutions were made up immediately prior to use, with the ammonium persulphate solution and the TEMED being added last. The resolving gel solution was pipetted between the glass plates separated by 1 mm spacers. The depth of the stacking gel between the bottom of the comb and the resolving gel was about 1 cm. Once the resolving gel had been poured it was layered with ethanol and allowed to polymerise for 30 min. The ethanol was then discarded and stacking-gel solution was poured on top of the resolving gel. The comb was inserted and polymerisation was allowed to occur. The comb was then removed and the wells washed out with

running buffer, which was also used to fill up the buffer chambers of the apparatus. The sample could be loaded onto the gel at this stage.

Samples were mostly whole-cell extracts. Bacteria were harvested by centrifugation in a microfuge tube, resuspended in an appropriate volume of 1×loading buffer (~100 µl) and boiled for 5 min. Gels were typically electrophoresed at a constant current of 20 mA per gel until the bromophenol blue dye-front had run off the bottom of the gel. Once electrophoresis was complete the glass plates were removed from the apparatus, separated carefully and proteins were analysed either by staining with Coomassie blue by Western blotting.

2.8.2. Staining of proteins with Coomassie blue

Solutions:

- staining solution

  10% (v/v) acetic acid, 50% (v/v) methanol, and 0.1% (w/v) Coomassie brilliant blue R250.

- destaining solution

  7% (v/v) acetic acid, and 5% (v/v) methanol.

Gels were separated from glass plates, transferred to a plastic box with staining solution and incubated on a shaker for 30 min at room temperature. The staining solution was replaced by destaining solution and two or three pieces of sponge were placed into the box to absorb Coomassie blue. Gels were incubated on a shaker until protein bands had become clearly seen (normally, it took 2-3h).

2.8.3. Purification of rabbit antiserum

Antibodies in rabbit antiserum (raised against *Eco*AI), that cross-reacted with *E. coli* proteins other than Hsd, were removed by readsorbing the preparations against a lysate of *E. coli* cells lacking of hsd_A (HfrH). Bacteria from 10 ml of stationary phase culture were harvested by centrifugation (3,000 × g, 5 min) and resuspended in 1 ml of TS (10 mM Tris-HCl pH 7.4, 150 mM NaCl). 100 µl of 10% (w/v) SDS was added, and the suspension was incubated for 5 min at 100°C. This lysate was diluted 10-fold in TS. Equal volumes of *E. coli* cell lysate and the antiserum were mixed by rotation for 12h at 4°C. Antibody-antigen complexes were removed by centrifugation

(17,000 × g, 15 min) at 4°C. The supernatant was transferred to a fresh microcentrifuge tube and an equal volume of cell lysate was added again. The procedure was repeated 4 times and the pre-adsorbed antiserum was stored at –20°C.

2.8.4. Western blotting

After protein gel electrophoresis was completed the apparatus was disassembled, one of the two glass plates was removed and the gel was covered with a PVDF membrane briefly wetted with methanol. No air bubbles should be left between the gel and the membrane. Three pieces of blotting paper soaked in transfer buffer (25 mM Tris, 190 mM glycine) were placed on top of the membrane. The other glass plate was removed and the gel was covered with three pieces of soaked blotting paper, i.e. the gel along with the membrane were between 6 pieces of blotting paper, three on either side. The "sandwich" was placed between two pads in a TransBlot Cell (BioRad) apparatus and the tank was filled with the transfer buffer. The proteins were transferred to the membrane by electrophoresis at 4°C for 1.5h at a constant voltage of 40V.

After electrophoresis the membrane was used for protein detection using the chemiluminescence detection system (POD) of Boehringer Mannheim. Through all the procedure the membrane was incubated on a shaker at room temperature. The membrane was blocked, to prevent non-specific adsorption of the antibodies during immunodetection, in block solution (a 1:10 dilution of the stock) in TBS buffer for 2h. The block solution was removed and replaced by solution with primary antibody (block diluted in TBS 1:20 and either 1:2500 of antiserum for *Eco*KI or 1:1000 of purified antiserum for *Eco*AI was added) and incubated for 2h. The membrane was washed 2 × 10 min in TBST buffer (0.1% Tween in TBS) and then 2 × 10 min in block solution (block diluted in TBS 1:20). The secondary antibody solution was added (block diluted in TBS 1:20 plus 1:1000 anti-rabbit IgG peroxidase conjugate) for 1h and then the membrane was washed 4 × 15 min in TBST. After the last wash the membrane was incubated with substrate solution for 1 min, covered with Saranwrap film and exposed to X-ray film for 3-30 sec.

2.8.5. *In vivo* protein stability assays

The stability of proteins was monitored following pulse-labelling with ^{35}S-methionine. Bacteria were grown at 37°C with intensive aeration to an OD_{600} of 0.2-0.3 in minimal medium supplemented with thiamine and all the amino acids except methionine and cysteine. Chloramphenicol (20 μg/ml) maintained the presence of pNK3. Each culture was divided and 2-AP (400 μg/ml) was added to one aliquot. After 1.5h a 1 minute pulse of ^{35}S-methionine (25 μCi/ml) was given. Labelling was stopped by diluting each culture with an equal volume of prewarmed L-broth supplemented with L-methionine (15 μM), or with L-methionine and 2-AP (400 μg/ml). Intensive aeration was maintained and samples were taken at appropriate intervals. Bacteria were collected by centrifugation, resuspended in SDS sample buffer and boiled for 5 min, and samples were applied to SDS-polyacrylamide gels for the separation of polypeptides by electrophoresis.

2.8.6. Assay of β-galactosidase

The assays were done following the procedure described by Miller (1972).

CHAPTER 3. ClpXP PROTEASE IS NECESSARY FOR THE EFFICIENT ACQUISITION OF TYPE IA AND IB RESTRICTION-MODIFICATION SYSTEMS.

3.1. Introduction

Genes for many R-M systems can be transferred readily from one strain to another in one step by conjugation, transformation or P1 transduction (Boyer, 1964; Colson & Colson, 1972; Sain & Murray, 1980; Bullas *et al.*, 1980; Fuller-Pace *et al.*, 1985; Suri & Bickle, 1985; Redaschi & Bickle, 1996; Kulik & Bickle, 1996). Experiments, quantifying such a transfer, find no evidence for any barrier to the acquisition of *hsd* genes by P1 transduction (O'Neill *et al.*, 1997). Early studies with phage P1 R-M activities (a type III system) demonstrated that the modification activity was already detectable a few minutes after infection of *E. coli* with P1 whereas restriction activity attained its normal level only some hours later (Arber, 1974). Some R-M systems of type I and III have been shown to delay expression of restriction activity up to 15 generation after *r-m* genes enter naive cells (Prakash-Cheng & Ryu, 1993; Prakash-Cheng *et al.*, 1993; Kulik & Bickle, 1996; Redaschi & Bickle, 1996). Apparently, bacteria possess a regulatory mechanism that enables them to survive the establishment of the new functions. This mechanism was implicated in the sequential establishment of modification and restriction, i.e the restriction activity is expressed only after the chromosome becomes modified.

The establishment of some type I systems is affected by the amount of HsdR in the cells prior to the establishment. When *hsdR* from type IA or IB system was expressed on a multicopy plasmid, $hsdR^{+}M^{-}S^{-}$ cells died upon upon acquisition of $hsdM^{+}S^{+}$ (Fuller-Pace *et al.*, 1985; Suri & Bickle, 1985; Kelleher *et al.*, 1991). Similar experiments for *Eco*R124I (IC family) revealed different results; bacteria survived the establishment of the new specificity (Kulik & Bickle, 1996). This finding implies either different or additional mechanisms of regulation for *Eco*R124I that can be provided by plasmid or host encoded functions or by both.

Although *hsdR* and *hsdMS* for type I are transcribed from different promoters, p_{res} and p_{mod} respectively, an effective regulation at the transcriptional level has not been

detected for the systems investigated (Loenen *et al.*, 1987; Kulik & Bickle, 1996). *lacZ*-fusions with the promoters of hsd_KR and hsd_KMS revealed no difference in β-galactosidase expression when these fusions were transferred to modification-deficient recipients by conjugation (Prakash-Cheng *et al.*, 1993). Simultaneous activity of both promoters during the establishment of the *Eco*KI system implies that the regulation at the level of transcription is not involved in the sequential establishment of the modification and restriction activities.

It has been found for type IA systems that a host factor is involved in the control of restriction activity following conjugation. A derivative of *E. coli* C, *hsdC,* incapable of the establishment of the *Eco*KI system was isolated (Prakash-Cheng *et al.,* 1993). Conjugative transfer of F'$hsd_KR^+M^+S^+$ to this mutant occurs with a frequency 10^5 lower in comparison with F'$hsd_KR^-M^+S^+$, presumably, because the cells are killed following entry of the hsd^+ plasmid and the expression of the endonuclease activity while the resident chromosome is unmodified. The lethal effect of F'$hsd_KR^+M^+S^+$ transfer for *hsdC* cells was confirmed by a drop in the titre of the recipient bacteria during conjugation.

Low efficiency of transfer of a type IA system to the *hsdC* mutant has also been shown by P1 transduction experiments (O'Neill *et al.*, 1997). The frequency of co-transduction of *dnaC-hsd* to *E. coli* K-12 is about 50%. However no $r_D^+m_D^+$ recombinant among 100 $dnaC^+$ transductants was obtained for the *hsdC* recipient, while the wild-type *E.coli* C revealed about 20% linkage. A similar co-transduction frequency between $DnaC^+$ and m_D^+ was observed for the transfer of $hsd_DR^-M^+S^+$ irrespective of the *hsdC* allele.

Conjugative transfer of the *hsd* genes of the *Eco*AI system (type IB) to *hsdC* recipients led to similar results (Kulik & Bickle, 1996). A decrease in the frequency of F'$hsd_AR^+M^+S^+$ transfer and recipient killing have been observed. However, both restriction and modification activities were found in transconjugants. No difference in the level of restriction in $hsdC^+$ and $hsdC^-$ cells of *E. coli* C has been shown for both *Eco*AI (Kulik & Bickle, 1996) and *Eco*KI (N. Murray, pers. commun.). HsdC seems to be a modulator of the restriction activity of the type IA and IB systems at least at the stage of establishment, but not for type IC: hsd^+_{R124I} can be transferred easily to the *hsdC* recipient (Kulik & Bickle, 1996).

3.2. Identification of the *hsdC* mutation

Rough mapping of the *hsdC* mutation led to the conclusion that the *hsdC*$^+$ allele of *E. coli* K-12 was between 6′ and 16′ of the chromosome map (Prakash-Cheng *et al.*, 1993). The HfrH strain, which has its origin of transfer (*oriT*) at 96.5′ and transfers the chromosome clockwise was used as a donor to map *hsdC* within a shorter fragment. The mapping relied on analysis of linkage between three markers two of which are already mapped (*leu* at 1.7 min and *lac* at 7.8 min), and the third one (*hsdC*) to be localised relative to the other two. A derivative of the *hsdC* strain containing *leu*::Tn*10* and a spontaneous *lac* mutation (NK84) was selected; the donor strain (HfrH) was different from the recipient (NK84) in three markers: *leu*$^+$*lac*$^+$*hsdC*$^+$ donor and *leu*$^-$*lac*$^-$ *hsdC*$^-$ recipient.

The test for the HsdC phenotype relied on the ability of HsdC$^+$ but not HsdC$^-$ cells to survive the establishment of the *Eco*KI specificity (Prakash-Cheng *et al.*, 1993), i.e. to acquire F′101-101 (*hsdR*$^+$*M*$^+$*S*$^+$) with the same efficiency as F′101-102 (*hsdR*$^-$*M*$^+$*S*$^+$). These plasmids included Tn*10* which permitted selection for Tet^r transconjugants. Because of the *leu*::Tn10 marker in the recipient, tetracycline could not be used to select transconjugants of NK84. Therefore, miniTn*5*-Cm insertion derivatives of F′101-101 and F'101-102 (F′101-201 and F′101-202 respectively) were constructed (see Materials and Methods) and chloramphenocol was used to select for transconjugants.

100 Leu$^+$ (proximal marker) recombinants obtained from the mating HfrH × NK84 were screened for both ability to utilise lactose and for their HsdC phenotype by testing their ability to acquire F′101-201 and F′101-202 in replica plating matings. F'101-201 contains *hsd*$_K$*R*$^+$*M*$^+$*S*$^+$ genes, therefore it can be transferred with high frequency to HsdC$^+$ but not to HsdC$^-$ cells. F'101-202 was used as a control; it can be acquired by both HsdC$^+$ and HsdC$^-$ recipients because the plasmid contains an *hsdR*$^-$ allele. The linkage between *leu*$^+$ and *lac*$^+$ was 17%, whereas only 14% of the Leu$^+$ recombinants exhibited an HsdC$^+$ phenotype (Fig.3.1). All the Leu$^+$HsdC$^+$ colonies were Lac$^+$. These results suggest that the order of the genes is *leu-lac-hsdC*. Analysis of 100 Lac$^+$ recombinants revealed 80% linkage between *lac*$^+$ and *hsdC*$^+$.

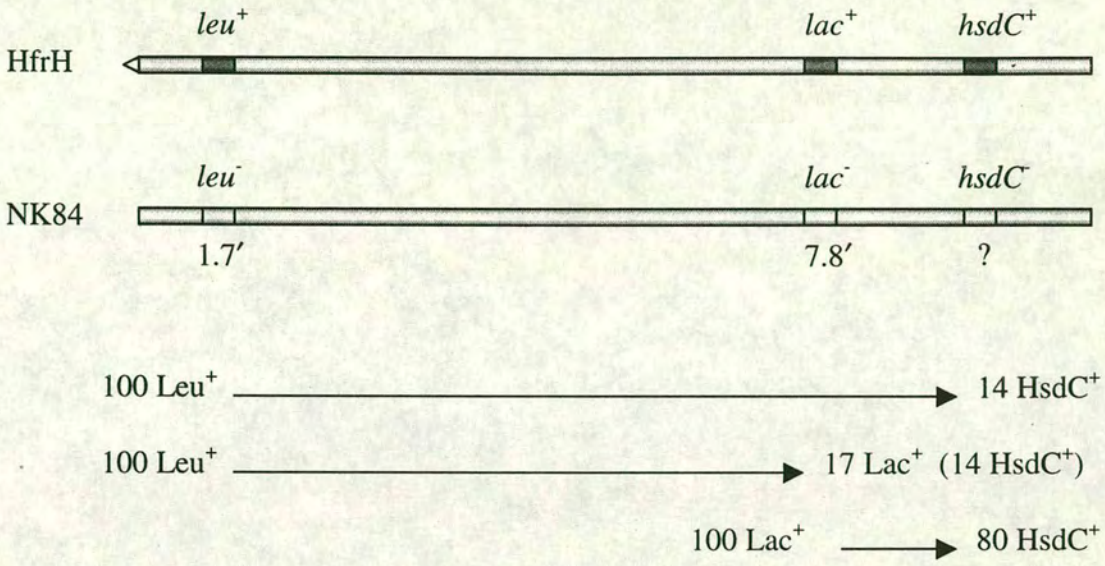


Figure 3.1. Analysis of recombinants from the conjugation of Hfr H × NK84 (See text for details).

The linkage data were used to estimate an approximate distance between *lac* and *hsdC* according to Jacob & Wollman (1961). The distance l between a proximal marker A and a distal marker B is $-k \ln x$, where x is a linkage between the markers when selected for the proximal one and k is a constant value within any conjugative system, i.e.

$$l = -k \ln x.$$

$$-k = l / \ln x, \text{ or}$$

$$l_1/\ln x_1 = l_2 / \ln x_2 = \ldots = l_n/\ln x_n.$$

Three pairs of markers can be considered in the experiment described: *leu-lac* (l_1 = 6.1′, $x_1 = 0.17$), *leu-hsdC* ($x_2 = 0.14$) and *lac-hsdC* ($x_3 = 0.80$).

$$6.1/\ln 0.17 = l_2/\ln 0.14 = l_3/\ln 0.8,$$

$$l_2 = 6.79′ \text{ (}leu\text{-}hsdC\text{ distance)},$$

$$l_3 = 0.76′ \text{ (}lac\text{-}hsdC\text{ distance)}.$$

This calculations place *hsdC* in a close proximity to *lac* (about 1 minute of chromosomal map).

P1 transduction experiments were used for further mapping. Some strains containing Tn*10* insertions within 8-11 min of the *E. coli* K-12 chromosome (Singer *et al.*, 1989) were crossed as donors with a Leu^+Tet^s derivative of NK84 (NK85). Tet^r transductants were screened for the HsdC phenotype as described before. The $hsdC^+$ allele showed 73% linkage with *zba-3054*::Tn*10* located at 10.1′ and 50% with *tsx-247*::Tn*10* (9.3') and therefore *hsdC* maps within the 9.3′-10.1′ segment of the *E. coli* K-12 chromosome.

It has been suggested that proteases could regulate the sequential establishment of the modification and restriction activities of type III (Redaschi & Bickle, 1996) and type I (Dryden *et al.*, 1997) systems. Genes encoding structural components of three proteases Lon, ClpXP and ClpAP are within the 9.3′-10.1′ region. It was supposed that *hsdC* could be a mutation in *clpP*, *clpX* or *lon*. The *Bam*HI fragment containing *bolA, tig, clpP* and *clpX* was cloned in the λ vector NM1151 (Murray, 1983) from the Kohara phage 3B6 [148] (Kohara *et al.*, 1987) and the resultant phage λNM1357 was used to make a lysogen of NM820 *hsdC* $recA^+$. The lysogen was tested for HsdC in conjugation with donors of F′101-201 and F′101-202 and was found to be $HsdC^+$.

To identify the gene mutated in the *hsdC* cells, phages similar to that described above but with mutations in *clpP*, *clpX* or in both genes were constructed and used to make

lysogens of NM820. The HsdC phenotype depended on the nature of the *clpX* allele on the phages: only $clpX^+$ phages restored efficient transfer of $hsd_KR^+M^+S^+$ to *hsdC* cells. Hence *hsdC* cells have a mutation in the *clpX* gene.

3.3. Analysis of *clpX* and *clpP* derivatives of *E.coli* K-12

JR302, the strain defined by Prakash-Cheng and co-workers as *hsdC* (Prakash-Cheng *et al.*, 1993), is a hybrid of *E. coli* C and *E. coli* K-12. Recombination following conjugation involves long fragments of chromosomal DNA and in the absence of col-linearity between donor and recipient genomes, recombination could lead to unknown rearrangements. Investigation of the effect of known mutations in an *E. coli* K-12 background was considered preferable to an analysis of JR302 and its derivatives. A *gyrA ΔhsdRM* derivative of the *E. coli* K-12 strain C600 was made (NM840) and used as a parent strain for constructing cells deficient in the ClpXP protease. However, JR302 and its derivatives were included as controls in further experiments to monitor the establishment of the *hsd* genes.

3.3.1. *clpX* and *clpP* bacteria are deficient in the establishment of new type IA and IB specificities.

$HsdC^-$ bacteria have been shown to die upon the acquisition of genes specifying functional *Eco*KI (type IA) or *Eco*AI (type IB) by conjugation (Prakash-Cheng *et al.*, 1993; Kulik & Bickle, 1996) as well as *Eco*DI (type IA) by P1 transduction (O'Neill *et al.*, 1997). *clpX*, which was found relevant to the $HsdC^-$ phenotype, encodes a component of the ClpXP protease. Both *clpX* and *clpP* cells are deficient in the active protease. Therefore the effect of both *clpX* and *clpP* on the transfer of the *hsd* genes encoding the *Eco*KI and *Eco*AI systems, by conjugation, transformation and P1 transduction, was investigated.

Conjugation

I. Strain construction. F'101-101 (Prakash-Cheng *et al.*, 1993) is a *zjj::*Tn*10* insertion derivative of F'101 (Low, 1972), a plasmid with a segment of *E. coli* K-12 chromosome (hsd_K^+) covering the *hsd* locus. The transposon insertion allows the use

of tetracycline to select for transconjugants. F'101-102 is an *hsdR* derivative of F'101-101. MiniTn*5*-Cm insertions were introduced to both plasmids to provide an additional selective marker. Therefore F'101-201 and F'101-202 confer both Tet^r and Cm^r.

F' plasmids similar to F'101-201 and F'101-202 but containing $hsd_AR^+M^+S^+$ and $hsd_AR^-M^+S^+$ (F'101-301 and F'101-302 respectively) were constructed. F'101 was transferred by conjugation to $r_A^+m_A^+$ (NK167) and $r_A^-m_A^+$ (NK170). In both strains the *hsd* genes were linked to the *zjj::*Tn*10* (Tet^r) insertion. Because F`101 contains a chromosomal segment of the *E. coli* K-12 genome overlapping the *hsd-zjj::*Tn*10* region recombination between chromosomal and plasmid DNA can occur and result in the transfer of the hsd_A-*zjj::*Tn*10* locus to the plasmid. To select such recombinant plasmids NK167 (F'101) and NK170 (F'101) were conjugated with *recA rpsL* bacteria (JC9935). Tet^rStr^r conjugants were tested for restriction and modification and the ability to transfer tetracycline resistance and hsd_A genes as expected for plasmid encoded markers in conjugation. MiniTn*5*-Cm derivatives of the plasmids constructed were selected as described before (see Materials and Methods).

ΔclpP::cat and *ΔclpX::kan* mutations were transferred by P1 transduction to the *E.coli* K-12 strain NM840 (*gyrA ΔhsdRM*) to create NK121 (NM840 *clpP*) and NK123 (NM840 *clpX*).

A *recA* strain, JC9935, was used in the conjugation experiments as a donor of the plasmids to prevent recombination between chromosomal and plasmid DNA in the donor strain and the consequent early conjugative transfer of clp^+ alleles to recipients.

II. Conjugation experiments. NK121, NK123, NM820 (JR302 $recA^+$) and NM840 and JR300 *gyrA* (JR300 is a wild-type of *E. coli* C) as controls were conjugated with donors of hsd_K and hsd_A (Table 3.1) at a ratio 1:10 (recipient : donor). Transfer of functional R-M genes, either hsd_K or hsd_A, resulted in a decrease in the titre of recipient bacteria defective in the ClpXP protease (as well as *hsdC* cells) (Table 3.1 and Fig.3.2 and 3.3).

Table 3.1.
Conjugative transfer of hsd_K and hsd_A genes to recipients deficient in ClpXP protease

Recipient strains	Donor strains					
	a. for *Eco*KI					
	JC9935 (F′101-202)[a]		JC9935 (F′101-201)[a]			
	Titre of recipients after conjugation ml^{-1}	Titre of Tet^r conjugants[b] ml^{-1}	Titre of recipients after conjugation ml^{-1}	Titre of Tet^r conjugants[b] ml^{-1}	Titre of Tet^r r^+m^+ conjugants ml^{-1}	Relative frequency (%) of survival of recipients[c]
NM840 (C600 *Δhsd gyrA*)	2.1×10^8	2.1×10^8	2.1×10^8	2.1×10^8	2.1×10^8	100.0
NM840 *clpP*	1.6×10^8	1.6×10^8	1.8×10^5	7.4×10^4	3.7×10^4	0.1
NM840 *clpX*	1.4×10^8	1.4×10^8	8.5×10^4	4.8×10^3	9.6×10^1	0.03
JR300 *gyrA* (*E. coli*C)	1.5×10^8	1.5×10^8	1.5×10^8	1.5×10^8	1.5×10^8	100.0
NM820 (*E. coli*C *hsdC*)	2.0×10^8	2.0×10^8	2.6×10^4	4.8×10^3	$<8.4\times10^1$	0.01
	b. for *Eco*AI					
	JC9935 (F′101-302)[d]		JC9935 (F′101-301)[d]			
NM840 (C600 *Δhsd gyrA*)	6.0×10^7	5.9×10^7	6.0×10^7	6.0×10^7	6.0×10^7	100.0
NM840 *clpP*	3.3×10^7	3.1×10^7	6.2×10^5	1.6×10^5	1.5×10^5	1.9
NM840 *clpX*	5.1×10^7	4.0×10^7	1.3×10^5	2.8×10^4	2.7×10^4	0.3
JR300 *gyrA* (*E. coli*C)	1.7×10^8	1.7×10^8	1.0×10^8	7.1×10^7	7.1×10^7	58.8
NM820 (*E. coli*C *hsdC*)	1.8×10^8	1.7×10^8	9.1×10^6	5.5×10^6	5.5×10^6	5.1

[a] F′101-202 and F′101-201 are derivatives of F′101-102 and F′101-101 respectively (Prakash-Cheng *et al.*, 1993) with miniTn*5*-Cm insertions (de Lorenzo *et al.*, 1990). The former includes $hsd_KR^-M^+S^+$ the latter $hsd_KR^+M^+S^+$.

[b] Nalidixic acid was used to select against the donor, with the exception of NM820 where kanamycin was used. Tetracycline was used to select for transconjugants.

[c] Frequency (%) with which recipient cells survived conjugation with the donor of F′101-201 and F′101-301 is expressed relative to that for F′101-202 and F′101-302 respectively [the ratio of the titre of recipient cells after conjugation with JC9935 (F′101-201) to the titre of recipient cells after conjugation with JC9935 (F′101-202) as a percentage; and the ratio of the titre of recipient cells after conjugation with JC9935 (F′101-301) to the titre of recipient cells after conjugation with JC9935 (F′101-302) as a percentage respectively].

[d] F′101-302 and F′101-301 are derivatives of F′101 (Low, 1972) where hsd_K^+ genes were replaced by $hsd_AR^-M^+S^+$ and $hsd_AR^+M^+S^+$ respectively. Both plasmids have *zjj*::Tn*10* and miniTn*5*-Cm.

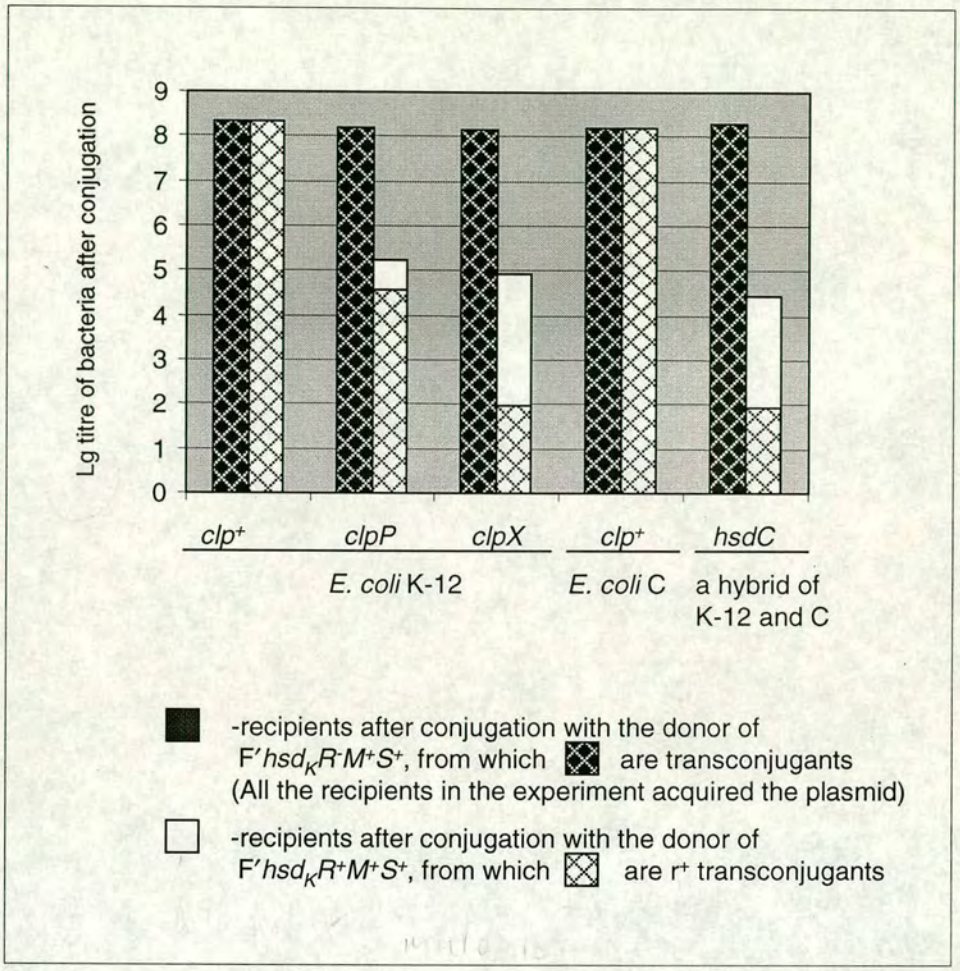


Figure 3.2. The effect of *clpX* and *clpP* on survival of recipient cells upon acquisition of $hsd_KR^+M^+S^+$ by conjugation.

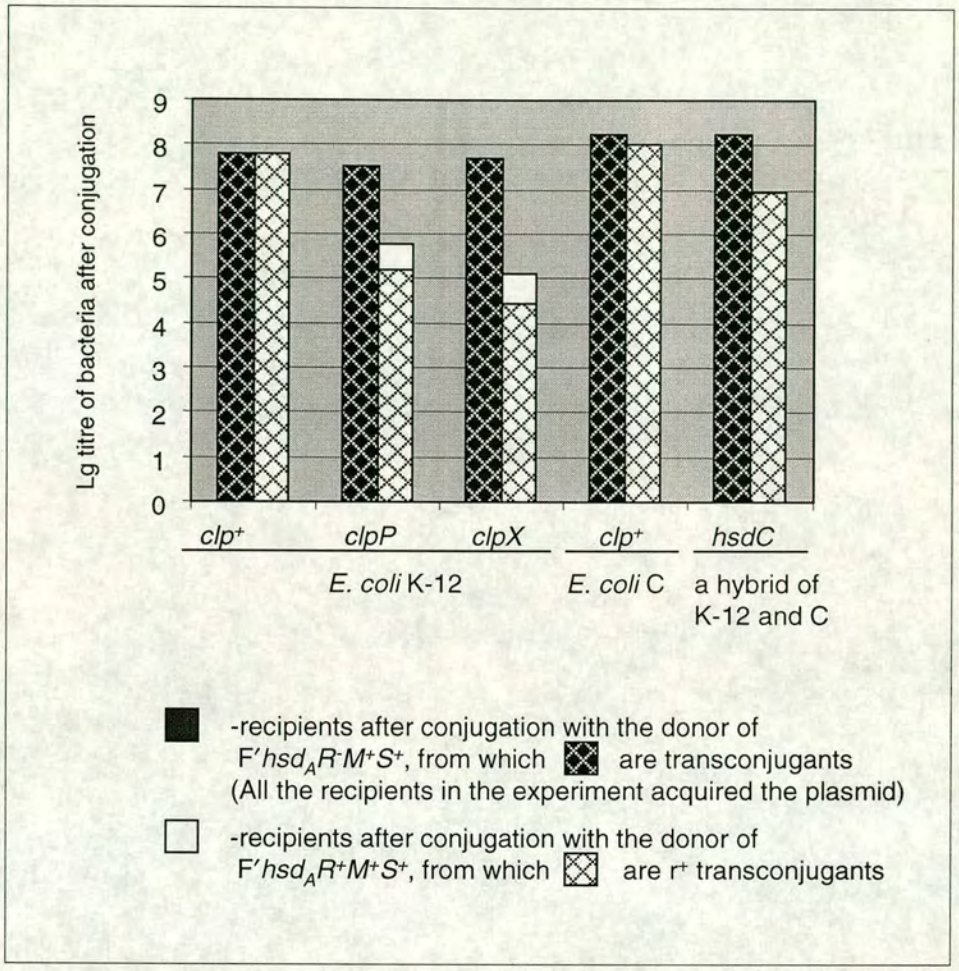
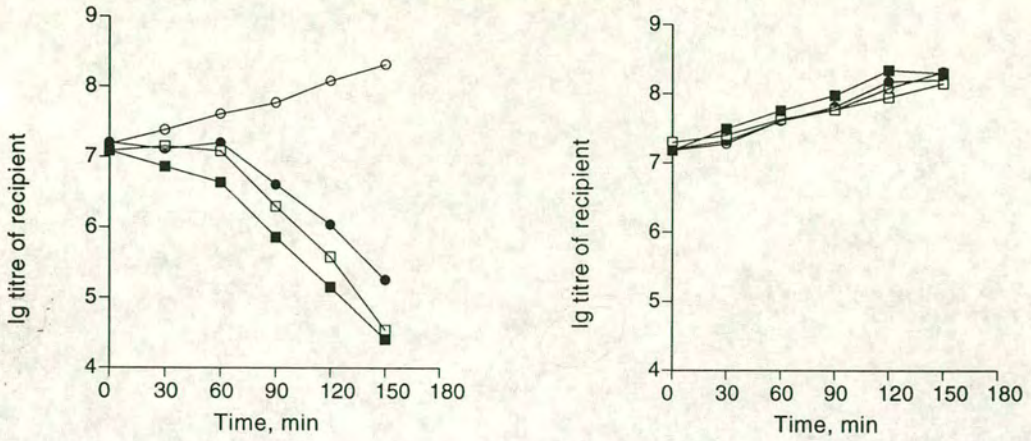


Figure 3.3. The effect of *clpX* and *clpP* on survival of recipient cells upon acquisition of $hsd_AR^+M^+S^+$ by conjugation.

Samples of conjugants presumed to have obtained the *hsdR*$^+$*M*$^+$*S*$^+$ genes were purified and tested for restriction. In the case of *Eco*AI 90-100% bacteria from both experimental and control matings displayed the r$^+$ phenotype. The absence of ClpXP was found to be a stronger barrier to the acquisition of the *Eco*KI system. For NK123 (*clpX*) only 2 conjugants out of 100 were r_K^+, for *clpP* 50% were r_K^+, whereas 100% of conjugants in the *clp*$^+$ controls (NM840 and JR300 *gyrA*) were restriction proficient. The effect of the *hsdC* mutation was at least as severe as *clpX*: none of 57 colonies tested expressed restriction activity, but 2 of them were $r_K^- m_K^+$. Most of the conjugants were sensitive to the male specific phage M13, an indication of the presence of an F′. F′s from r$^+$m$^+$ and r$^-$m$^-$ *clpX* bacteria and from r$^-$m$^+$ and r$^-$m$^-$ *hsdC* conjugants were transferred into NM840 (through JC9935 in the case of *clpX* cells because no counterselection was available for the direct mating) and the Tet^{r}Nal^r conjugants exhibited r$^+$m$^+$, r$^-$m$^-$, r$^-$m$^+$ and r$^-$m$^-$ phenotypes respectively. Apparently, r$^-$ conjugants acquired plasmids that were no longer hsd_K^+. This can be explained by the formation of mutant derivatives of F′101-201 in the original donor strain JC9935. Such plasmids have lost *hsd* genes or have an *hsd* mutation but have retained the chromosomal fragment with the Tn*10* insertion. They can be transferred into the *clp* mutants not only from JC9935 but can readily spread in the population by the secondary donors - the recipient cells acquiring the plasmids from the original donors. The conditions, in which the acquisition of the *hsd* genes caused cell death, led to selection of such defective variants of F′101-201. Therefore the frequency of formation of Tet^{r}Nal^r (Tet^{r}Kan^r for NM820) conjugants did not coincide with the real frequency of *hsd* genes transfer that was reflected in the table 3.1 and figures 3.2 and 3.3. The difference in checks on Tet^r transconjugants formed in matings of the *clp* mutants with a donor of *hsdR*$^+$*M*$^+$*S*$^+$ and with a donor of appropriate *hsdR*$^-$*M*$^+$*S*$^+$ genes was even greater than that for the titre of recipients that survived conjugation.

The dynamics of killing of *clpP* and *clpX* mutants during conjugation with JC9935 (F′101-201) is shown in fig.3.4. Control matings with JC9935 (F′101-202) revealed no drop in titre of any of the recipient bacteria tested.



a. b.

Figure 3.4. Conjugative transfer of (a) $hsd_KR^+M^+S^+$ and (b) $hsd_KR^-M^+S^+$genes to NM840 (○), NM840 *clpP* (●), NM840 *clpX* (□) and NM820 *E. coli* C *hsdC* (■). Cultures of donor [JC9935 (F'101-201) and JC9935 (F'101-202)] and recipient bacteria, grown to mid-log phase, were mixed at a ratio of 10:1 and samples were plated at 30 min intervals on media selective for recipients (nalidixic acid for NM840, NM840 *clpP* and NM840 *clpX* and kanamycin for NM820).

Table 3.2. The effect of *clpP* and *clpX* on uptake of *hsd* genes by transformation

Recipient strains	Ratio of transformants with *hsd* plasmids to control plasmid			
	p*hsdRMS*$_K$ (pBE3)	p*hsdMS*$_K$ (pBE3*)	p*hsdRMS*$_A$ (pFFP30)	p*hsdMS*$_A$ (pFFP31)
NM840 (C600 *Δhsd gyrA*)	3.0	10.9	9.5	6.9
NM840 *clpP*	6.7×10^{-2}	6.6	1.7	5.6
NM840 *clpX*	6.6×10^{-3}	10.3	2.3	7.3
JR300 (*E. coli*C)	2.4	6.8	4.3	5.8
NM820 (*E. coli*C *hsdC*)	$<10^{-4}$ [a]	5.3	6.0×10^{-1}	8.7

[a] 0 Amp^r transformants isolated.

Transformation

The efficiency of transformation depends on the competence of the cells, and conditions of treatment that can vary from experiment to experiment. This influence can be overcome by estimating the relative efficiency of transformation when a mixture of two plasmids (a marker plasmid and a test plasmid) is used for transformation. The marker plasmid contains *kan* and *tet*, genes which are presumed to have no effect on the cell survival. Therefore the ratio of bacteria with the test plasmid to those with the marker plasmid reflects the effect of the acquisition of the test plasmid on cell survival.

A marker plasmid pBRK was constructed to estimate the relative efficiency of transformation of clp^+ and clp^- bacteria with plasmids containing hsd_K and hsd_A genes. pBRK is a derivative of pBR322 where *amp* is inactivated by the insertion of *kan* in the *Pst*I site. The resulting plasmid confers $Kan^rTet^rAmp^s$ whereas the plasmids with *hsd* genes (pBE3, $hsd_KR^+M^+S^+$; pBE3*, $hsd_KR^-M^+S^+$; pFFP30, $hsd_AR^+M^+S^+$ and pFFP31, $hsd_AR^-M^+S^+$) are Tet^sAmp^r. A mixture of DNA of two plasmids, one of which was always pBRK and the other one contained *hsd* genes, was used to transform clp^+, *clpP, clpX* and *hsdC* bacteria all of which were deficient in restriction and modification. The cells were made competent by electroporation. Ampicillin and tetracycline resistant transformants were selected to score the ratio of Amp^r colonies to Tet^r ones. For p$hsdR^-M^+S^+$/pBRK experiments this ratio did not depend on the state of the *clpPX* alleles but transformations with p$hsdR^+M^+S^+$/pBRK revealed a significant drop in the efficiency of formation of Amp^r transformants in the case of *clpP* and *clpX* mutants in comparison with clp^+ bacteria (Table 3.2). Thus the ClpXP protease affects the establishment of the *Eco*KI and *Eco*AI systems transferred by transformation. The *hsdC* mutant showed a more severe effect on the acquisition of both the *Eco*AI and *Eco*KI systems than either *clpP* or *clpX* derivatives of *E. coli* K-12.

P1 transduction

P1 transduction is a commonly used method for strain construction including the transfer of different *hsd* alleles from one background to another. An hsd^+ allele can be easily replaced for *Δhsd* and vice versa. It has been shown that neither acquisition nor elimination of a type I restriction-modification system is lethal for a cell, but an *hsdC*

mutation prevents the acquisition of $hsd_DR^+M^+S^+$ (type IA system) by P1 transduction (O'Neill *et al.*, 1997). The effects of *clpP* and *clpX* on uptake of hsd_K by P1 transduction were assessed.

As a preliminary step a $dnaC^{ts}$ (*dnaC325*) mutation was transferred by P1 transduction to the chromosome of the restriction-modification deficient $clpP^+X^+$ bacteria (NM840) as a marker closely linked with *zjj*::Tn*10*, an insertion that provided selection of recombinants. *clpP* (NK122) and *clpX* (NK124) derivatives of the resultant strain (NK125) were made by P1 transduction. NK125, NK122 and NK124 were transduced with P1 (C600 $hsdR^+M^+S^+$) and P1 (5K $hsdR^-M^+S^+$) to select $DnaC^+$ cells at 42^0C. The recombinants were screened for Tet^r as well as for restriction in the first set of crosses and for modification in the second.

Transfer of the $hsd_KR^+M^+S^+$ allele revealed significant differences in the linkage of *dnaC* to *hsd* between the parent clp^+ strain and its *clpP* and *clpX* derivatives, while $hsd_KR^-M^+S^+$ was co-transduced with $dnaC^+$ at a similar frequency irrespective of the *clpP* and *clpX* alleles (Table 3.3). The native state of general recombination in *clp* derivatives was confirmed by the similarity in $dnaC^+$-Tn10^0 (Tet^s) linkage in all P1 transduction experiments. The decrease in $dnaC^+$- $hsd_KR^+M^+S^+$ linkage in the cells deficient in the protease can be explained by the killing effect observed in the conjugation experiments. In the absence of the ClpXP protease most bacteria acquiring the functional hsd_K genes did not survive, presumably expression of these genes resulted in the formation of the active endonuclease while the chromosome was still unmethylated.

Similar experiments investigating the effect of *clpP, clpX* and *hsdC* on transfer of hsd_A genes by P1 transduction revealed no difference in the frequency with which $hsd_AR^+M^+S^+$ were co-transduced with $dnaC^+$ in either clp^+ and clp^- or $hsdC^+$ and $hsdC^-$ recipients (Table 3.4). Both in conjugation and in transformation, the Clp^- as well as the $HsdC^-$ phenotype resulted in a stronger barrier for *Eco*KI than for *Eco*AI. P1 transduction experiments showed qualitative difference in the process of acquisition of type IA and type IB R-M systems by the *clp* mutants.

Table 3.3. The effect of *clpP* and *clpX* on uptake of hsd_K genes by P1 transduction

Recipient strains	Donor strains					
	C600($hsd_KR^+M^+S^+$ *dnaC*$^+$)			5K ($hsd_KR^-M^+S^+$ *dnaC*$^+$)		
	Number of recom-binants tested	*dnaC*$^+$ - hsd_K^+ linkage	*dnaC*$^+$ - Tn*10*^{0} (Tet^s) linkage	Number of recom-binants tested	*dnaC*$^+$ - hsd_K *MS*$^+$ linkage	*dnaC*$^+$ - Tn*10*^{0} (Tet^s) linkage
NK125 (NM840 *dnaC zjj*::Tn*10*)	50	0.32	0.90	50	0.42	1.00
NK125 *clpP*	100	0.09	0.96	100	0.31	0.96
NK125 *clpX*	100	0.00	0.94	50	0.46	0.98

Table 3.4. The effect of *clpP* and *clpX* on uptake of hsd_A by P1 transduction.

Recipient strains	Donor strains					
	WA2899($hsd_AR^+M^+S^+$*dnaC*$^+$)			NM863($hsd_AR^-M^+S^+$ *dnaC*$^+$)		
	Number of recom-binants tested	*dnaC*$^+$ - hsd_K^+ linkage	*dnaC*$^+$ - Tn*10*^{0} (Tet^s) linkage	Number of recom-binants tested	*dnaC*$^+$ - hsd_K *MS*$^+$ linkage	*dnaC*$^+$ - Tn*10*^{0} (Tet^s) linkage
NK125 (NM840 *dnaC zjj*::Tn*10*)	60	0.48	0.83	50	0.54	0.90
NK125 *clpP*	100	0.63	0.88	49	0.51	0.81
NK125 *clpX*	100	0.65	0.80	49	0.63	0.84
NM824 (*E. coli* C *dnaC jj*::Tn*10*)	48	0.31[a]	0.75	48	0.33[a]	0.83
NM822 (*E. coli* C *hsdC dnaC zjj*::Tn*10*)	50	0.42[a]	0.90	48	0.42[a]	0.90

[a]. Lower frequencies of co-transduction were observed in similar experiments between *E. coli* K-12 and *E. coli* C (O'Neill *et al.*, 1997).

3.3.2. ClpXP is not involved in the establishment of the modification activity.

Prakash-Cheng & Ryu (1993) followed the establishment of both restriction and modification activities of *Eco*KI upon conjugative transfer of hsd_K^+ on an F′. They claimed that the modification activity was expressed immediately after the recipients had acquired the plasmid while the expression of the restriction activity took place much later. The delayed expression of restriction was implicated in sequential establishment of a new type I specificity: modification is established first and after the chromosomes becomes methylated the restriction activity appears. However, the method used for timing of modification is open to challenge (see the legend to Fig.3.5). ClpP$^-$ or ClpX$^-$ cells do not survive the establishment of a new specificity presumably because restriction is expressed when the bacterial chromosomes are still not modified. What is the role for ClpXP? Does it provide the quick establishment of the methylation activity or hold up the expression of restriction?

The effect of the *clpX* mutation on the establishment of the methyltransferase activity of *Eco*KI was tested in conjugation experiments (see Fig.3.5 for the method). It was found that the establishment of modification did not occur immediately but took about 12 hours, which in this particular experiment corresponded to 10 generations and was not influenced by the *clpX* allele (Fig.3.6). More intensive aeration of the cross mixture would probably increase the period of the establishment because the amount of DNA in exponentially growing bacteria with a short period of doubling corresponds to more than one genome per cell. Therefore the number of target sequences is increased. The chromosome replication not only increases the number of unmodified targets but also produces hemimodified target sequences which will distract the methylase from methylating unmodified DNA. On the other hand, replication might increase the *hsd* gene dosage that would compensate for the excessive amount of DNA. Bacterial growth followed by cell division can be considered as a factor of growing volume that makes it more difficult for the cells to accumulate the methylase and reach the complete methylation of the chromosome.

Probably, the ClpXP protease is involved in the establishment of the restriction activity delaying the production of a restriction-proficient phenotype relative to that of modification proficiency.

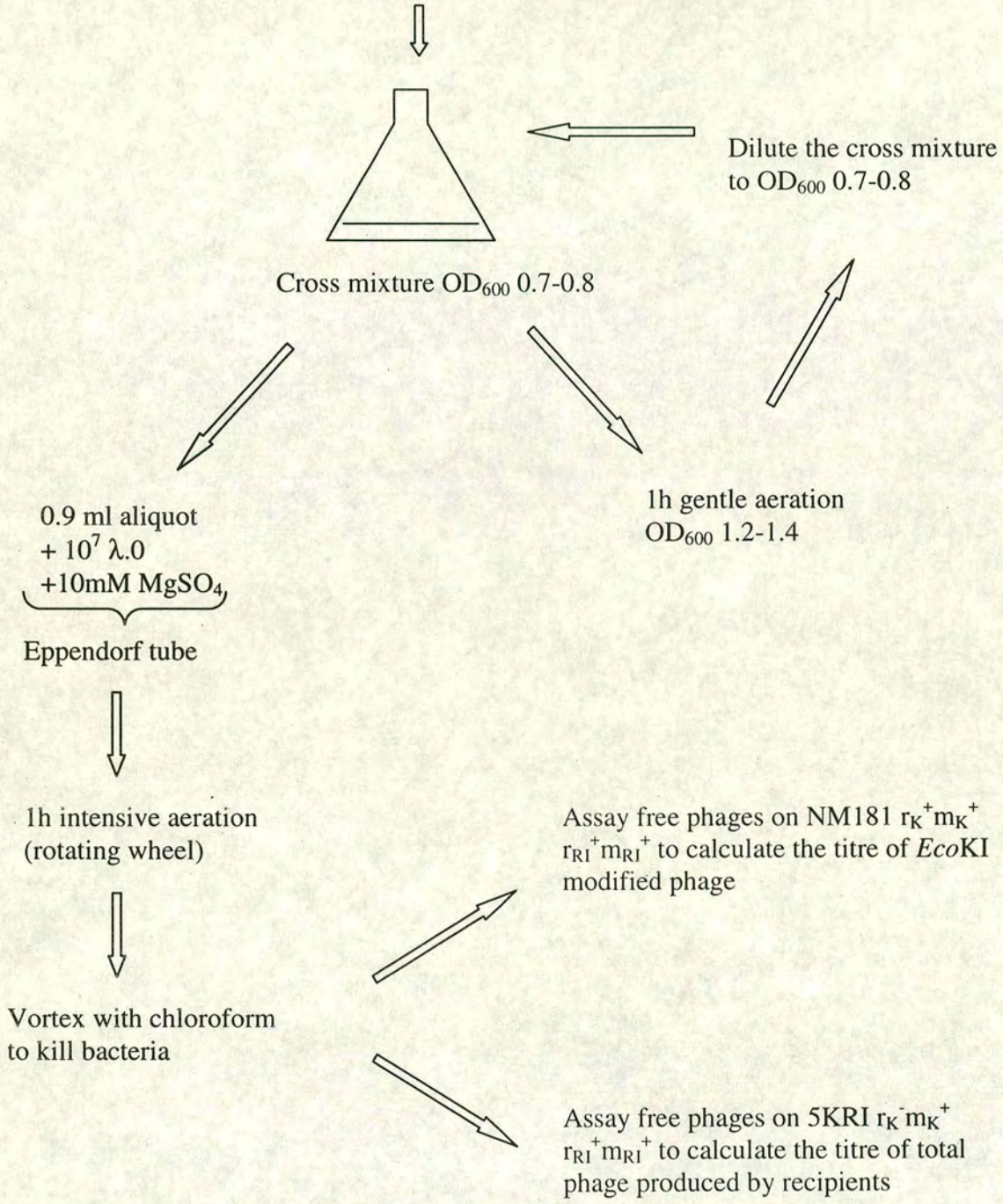

Dilute the cross mixture to OD_{600} 0.7-0.8
Cross mixture OD_{600} 0.7-0.8
1h gentle aeration OD_{600} 1.2-1.4
0.9 ml aliquot
+ 10^7 λ.0
+10mM $MgSO_4$
Eppendorf tube
1h intensive aeration (rotating wheel)
Vortex with chloroform to kill bacteria
Assay free phages on NM181 $r_K^+m_K^+$ $r_{RI}^+m_{RI}^+$ to calculate the titre of *Eco*KI modified phage
Assay free phages on 5KRI $r_K^-m_K^+$ $r_{RI}^+m_{RI}^+$ to calculate the titre of total phage produced by recipients

Figure 3.5 Assays for the establishment of the modification activity. Donor and recipient bacteria were grown till mid-log phase and mixed at a ratio 10:1 and the cross mixture was maintained in the log-phase by adding fresh prewarmed LB. Samples of the cross mixture were taken every hour and infected with λ.0. The donor cells had been made resistant to λ by introducing *malB*, therefore the phage could infect only the recipients. The phages were allowed to propagate for a single round and the progeny were tested for *Eco*KI-specific modification. The problem of unadsorbed phages present in the samples was overcome by having the *Eco*RI methyltransferase gene on a plasmid in the recipient cells: the λ propagated in the recipients become protected against *Eco*RI while unadsorbed phages do not. The *Eco*RI R-M system (in addition to *Eco*KI) present in the bacteria used as test cultures (NM181 and 5KR1) for assaying the efficiency of K-specific modification by the recipients restricts the unadsorbed phages. This method tests for the expression of modification within a time interval (1h in my experiments) used for a single round of infection with unmodified phage, while Prakash-Cheng & Ryu (1993) plated λ.0 on the samples of the cross mixture and assayed for modification of the phages within plaques. It takes about 6-8h for λ to form a plaque therefore many of the phages tested were produced by the transconjugants at least 6-8h later than expected.

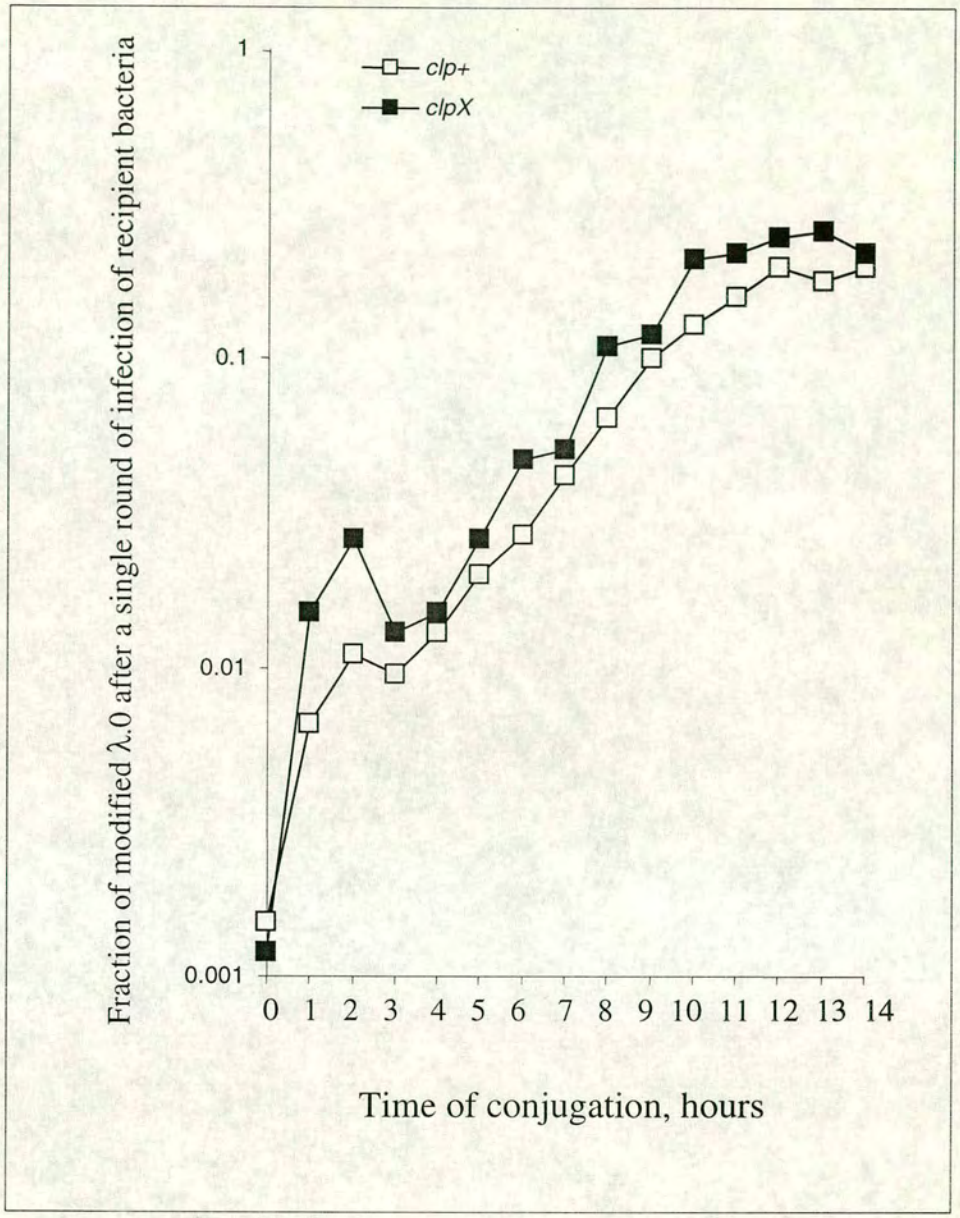


Figure 3.6. The establishment of the modification activity in *clp*$^+$and *clpX* cells following conjugative transfer of hsd_K.

3.3.3. ClpXP is not necessary to survive the loss of the *hsd* genes.

It has been shown that functional genes encoding type II R-M systems when present on a plasmid stabilise the maintenance of the plasmid (Naito *et al.*, 1995; Kulakauskas *et al.*, 1995). It was suggested that loss of the plasmid led to cell death. When cells, which have lost the plasmid continue to divide both the nuclease and the methyltransferase are diluted, and eventually the amount of residual methyltransferase becomes insufficient to protect the chromosome from residual nuclease.

Approximately 50% of *hsd* mutants of type I R-M systems obtained in random mutagenesis are r^-m^- (Wood, 1966): mutations in *hsdM* or *hsdS* result in lost of both endonuclease and methylase activities. This fact suggests that type I and type II systems behave in a different way and that the functional *hsd* genes can be lost without the cell being killed by residual nuclease. O'Neill *et al.* (1997) have quantified the deletion of hsd_K and have shown that there is no barrier to losing hsd_K even in *hsdC* cells.

P1 transduction was used to delete the hsd^+ allele in clp^+ and clp^- cells. The deletion mutants retained a functional *hsdR* gene ($hsd_KR^+M^-S^-$ or $hsd_AR^+M^+S^-$). The *Δhsd* allele was co-transduced with $dnaC^+$, as describe above, or with *zjj::*Tn*10* to restriction proficient clp^+, *clpP* and *clpX* recipients (Tables 3.5 and 3.6). Both $DnaC^+$ and Tet^r transductants were scored for restriction. No difference in the frequency of co-transduction of *Δhsd* with $dnaC^+$ or *zjj::*Tn*10* was found between clp^+ and clp^- cells (Tables 3.5 and 3.6). Both *Eco*KI and *Eco*AI specificities can be lost without any problem for the host, even in the absence of ClpXP. These results might imply the existence of additional mechanisms regulating the levels of the Hsd proteins in bacteria.

3.3.4. Expression of the restriction activity in ClpXP-deficient cells

Although both promoters p_{res} and p_{mod} of hsd_K are similarly active the amount of methylase M_2S_1 is much higher than the amount of endonuclease $R_2M_2S_1$ in wild-type cells (Weiserova *et al.*, 1993), i.e. there is insufficient HsdR polypeptide to convert all the methylase molecules to endonuclease. Based on the experiments showing that the ClpXP protease is relevant to the establishment of the *Eco*KI and *Eco*AI systems it was

Table 3.5.

The effect of *clpP* and *clpX* on elimination of *Eco*KI specificity by P1 transduction

Recipient strains	Donor strain NK231($hsd_KR^+M^-S^-$ *zjj*::Tn*10*)	
	Number of recombinants tested	*zjj*::Tn*10* (Tet^r) - $hsd_KR^+M^-S^-$ linkage
NK31 (C600 *gyrA*)	50	0.24
NK31 *clpP*	50	0.24
NK31 *clpX*	50	0.22

Table 3.6.

The effect of *clpP* and *clpX* on elimination of *Eco*AI specificity by P1 transduction

Recipient strains	Donor strain NM789 ($hsd_AR^+M^+S^-$ $dnaC^+$)		
	Number of recombinants tested	$dnaC^+$ - hsd_K MS^+ linkage	$dnaC^+$ -Tn10^0 (Tet^s) linkage
NM858 (WA2899 *dnaC zjj*::Tn*10*)	50	0.24	0.92
NM858 *clpP*	50	0.26	0.94
NM858 *clpX*	50	0.22	0.94

Table 3.7. The effect of *clpP* and *clpX* on expression of restriction activity.

Strains	Efficiency of plating (e.o.p.)[a]	
a. *Eco*KI system[b]	λ.0	λ.K
C600 *gyrA*	$(1.43 \pm 0.02) \times 10^{-4}$	0.75 ± 0.05
C600 *gyrA clpP*	$(2.03 \pm 0.85) \times 10^{-5}$	0.86 ± 0.29
C600 *gyrA clpX*	$(1.27 \pm 0.24) \times 10^{-5}$	0.95 ± 0.21
b. *Eco*AI system[c]	λ.0	λ.A
WA2899	$(1.25 \pm 0.11) \times 10^{-2}$	1.00 ± 0.25
WA2899 *clpP*	$(2.89 \pm 0.32) \times 10^{-3}$	0.87 ± 0.08
WA2899 *clpX*	$(1.84 \pm 0.17) \times 10^{-3}$	0.90 ± 0.10

[a] The data are based on three independent experiments.

[b] e.o.p. was calculated relative to a $\Delta hsd_K RM$ derivative of C600 *gyrA* (NM840).

[c] e.o.p. was calculated relative to an $hsd_A R^- M^+ S^+$ derivative of WA2899 (NM863).

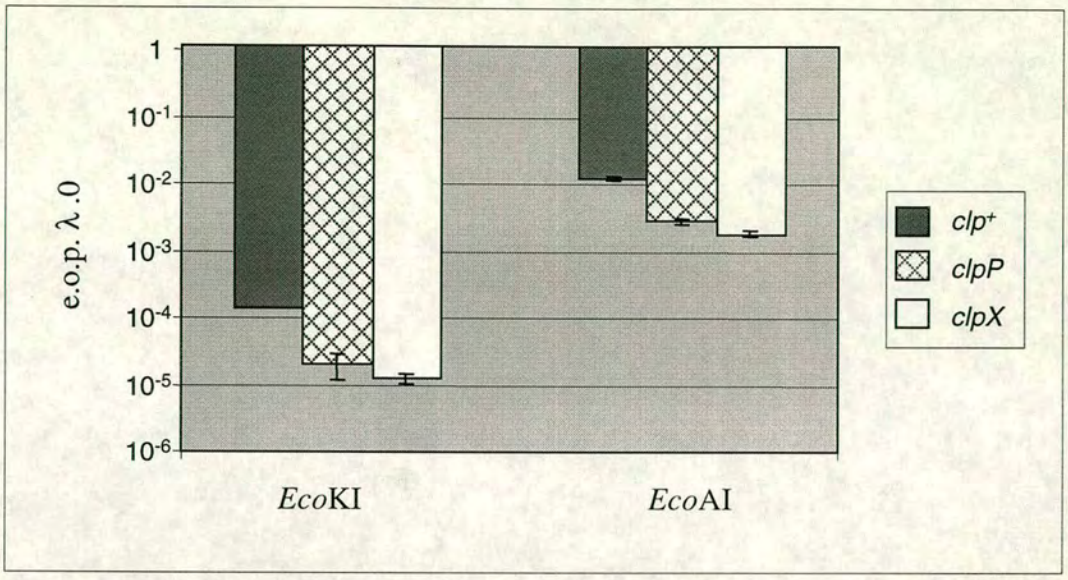


Figure 3.7. The effect of *clpP* and *clpX* on expression of restriction activity. For *Eco*KI the e.o.p. was calculated relative to a Δhsd_KRM derivative of C600 *gyrA* (NM840). For *Eco*AI the e.o.p. was calculated relative to an $hsd_AR^-M^+S^+$ derivative of WA2899 (NM863). Modified phage (λ.K or λ.A for the appropriate strains) plated with e.o.p. of 1 irrespective of the *clp* alleles.

suggested that HsdR could be degraded by the protease. In this case, cells deficient in ClpXP are expected to have increased levels of HsdR and enhanced restriction.

The efficiency of plating (e.o.p.) of unmodified λ on restriction proficient *clp*$^+$, *clpP* and *clpX* bacteria was assessed. For both *Eco*KI and *Eco*AI systems the *clp* mutations enhanced the levels of restriction about 4-10 fold compared to *clp*$^+$ cells (Table 3.7 / Fig. 3.7). The *clpX* defect had a slightly stronger effect than *clpP* on restriction activity. Similar results were obtained for AB1157, a *rac*$^-$ strain with an enhanced level of restriction (Webb *et al.*, 1996), and its *clp* derivatives (data not shown). However, the *hsdC* mutant showed completely different behaviour; once established in *hsdC* cells neither *Eco*AI nor *Eco*KI showed a difference in expression of nuclease activity in comparison with the *hsdC*$^+$ parent strain (Kulik & Bickle, 1996; Murray, pers. commun.).

3.4. Analysis of the *hsdC* mutant

According to the experiments described above the *hsdC* mutant behaved differently from either *clpP* or *clpX* derivatives of *E. coli* K-12. This difference was especially apparent in the transfer of the hsd_K genes by transformation. *hsdC* had a more severe effect than *clpX* in a C600 background, although it has been shown that *hsdC* cells have a mutation in *clpX*.

hsdC is believed to be a spontaneous mutation (Prakash-Cheng *et al.*, 1993). It was obtained from conjugation experiments to transfer the *recA* allele of CAG5055, an *E. coli* K-12 Hfr donor, to JR300, an *E. coli C* recipient. Similar experiments were performed to investigate the origin of such a mutant. *recA*::Cm was transduced into KL16 (CAG5055 is a derivative of KL16) and the resultant donor, KL16 *recA*::Cm, was conjugated with JR300 *gyrA*. 100 Cm^{r}Nal^r transconjugants were purified and screened for MMS sensitivity (*recA* cells are MMS^s), growth on minimal media and ability to acquire F′101-101 ($r_K^+m_K^+$) and F′101-102 ($r_K^-m_K^+$) in replica plating matings. Surprisingly, 45% of recombinants were MMS^r, as if they contained both *recA*$^+$ and *recA*::Cm at the same time. During further purification on L-agar with chloramphenicol and nalidixic acid they were unstable and gave two types of colonies: MMS^s and MMS^r. Tests for growth on minimal media revealed an auxotroph that

required tyrosine. Perhaps the gene order in the chromosome of *E. coli* K-12 is not absolutely identical to that in *E. coli* C. Recombination following conjugation between these strains might lead to some rearrangements of DNA including insertions and deletions.

Tests for the HsdC phenotype revealed a recombinant (NK105) which acquired F′101-202 but not F′101-201 in replica plating matings. A RecA$^+$ derivative of this newly obtained hybrid of *E. coli* K-12 and *E. coli* C (NK107) was characterised. It resembled *clpP, clpX* and *hsdC* mutants in that it died upon hsd_K transfer by conjugation, but it was different from the original *hsdC* mutant in transformation experiments. Some restriction proficient colonies of NK107 containing pBE3 $hsd_KR^+M^+S^+$ were isolated while no Amp^r colonies of NM820 (*hsdC*) were obtained following transformation with this plasmid.

Complementation with derivatives of λ containing *clp* genes was used to identify the mutation in NK107 (Table 3.8). Only lysogenisation with λNM1357$clpP^+X^+$ resulted in full restitution of the HsdC$^+$ phenotype. λNM1359$clpP^-X^+$ which complemented *hsdC* completely had no effect on NK107. λNM1361$clpP^+X^-$ reduced the killing effect of the mutation but cells still died upon conjugation with a donor of F′101-201. These results did not allow the identification of this mutation as *hsdC* but the defect was definitely caused by changes in the *clpPX* region because the phages containing $clpP^+X^+$ fully complemented the defect. Hence recombination following conjugation between *E. coli* K-12 and *E. coli* C might be a source of mutations in the *clpPX* region one of which has led to the *hsdC* derivative. If this mutation is a result of recombination then at least half a chromosome of the cell was involved in recombination because *recA*, a proximal marker transferred from the donor, is at 60′ of the map whereas the *clp* genes are at 10′. The *hsdC* mutant strain is a hybrid of *E. coli* K-12 and C with indefinite background that may contain additional mutations which affect the establishment of R-M systems.

To determine whether the difference between JR302 (*hsdC*) and NM840 *clpX* is relevant to the background or it reflects a particularity of the mutation within the gene, the *clpX* mutation of the *hsdC* cells ($clpX^{hsdC}$) was transferred to both *E. coli* C and *E. coli* K-12 bacteria. The *zba-3054*::Tn*10* insertion linked to the *clp* genes was introduced by P1 transduction into *hsdC* bacteria (NK85) and an HsdC$^-$ derivative

Table 3.8. Complementation of the HsdC$^-$ phenotype of NK107

Recipient strains	Donor strains				
	JC9935 (F'101-202)		JC9935 (F'101-201)		
	Titre of recipients after conjugation ml^{-1}	Titre of Tet^r conjugants[a] ml^{-1}	Titre of recipients after conjugation ml^{-1}	Titre of Tet^r conjugants[a] ml^{-1}	Relative frequency (%) of survival of recipients
NK107	9.2×10^7	6.8×10^7	4.0×10^4	1.0×10^3	0.04
NK107(λNM1357*clpP*$^+$*X*$^+$)	6.3×10^7	1.9×10^7	4.2×10^7	4.2×10^7	66.7
NK107(λNM1359*clpP*$^-$*X*$^+$)	1.7×10^7	6.0×10^6	1.0×10^4	2.0×10^2	0.06
NK107(λNM1361*clpP*$^+$*X*$^-$)	3.2×10^7	2.0×10^7	4.2×10^6	1.4×10^4	13.1

[a] Nalidixic acid was used to select against donor cells.

Table 3.9.

The effect of $clpX^{hsdC}$ in different backgrounds on uptake of hsd_K by transformation

Recipient strains	Ratio of transformants with *hsd* plasmids to control plasmid	
	p*hsdRMS*$_K$ (pBE3)	p*hsdMS*$_K$ (pBE3*)
JR300 (*E. coli* C)	9.7	6.8
NM840 (*E. coli* K-12 *Δhsd*)	12.8	7.7
NM820 (K-12 and C hybrid, $clpX^{hsdC}$)	$<10^{-4}$ [a]	5.3
NK176 (*E. coli* C $clpX^{hsdC}$)	2.7×10^{-3}	4.4
NK180 (*E. coli* K-12 $clpX^{hsdC}$)	7.5×10^{-2}	6.9

[a] 0 Amp^r transformants isolated.

(NK172) was selected. A P1 lysate grown on *hsdC zba-3054*::Tn*10* cells (NK172) was used to transfer the *clpX*^{hsdC} mutation as a marker linked to Tn*10* in *E.coli* K-12 NM840 and *E.coli* C JR300 *gyrA* backgrounds. In both experiments HsdC$^-$ derivatives found among Tet^r transductants were detected in replica plating matings with donors of F'101-201 and F'101-202.

Newly obtained *clpX*^{hsdC} mutants of *E. coli* C and *E. coli* K-12 (NK176 and NK180 respectively) were compared with the original *hsdC* bacteria NM820 (JR302 *recA*$^+$) in transformation experiments as described above (Table 3.9). Because NK176 and NK180 were Tet^r kanamycin was used to select for bacteria that inherited pBRK. The null mutation *ΔclpX::kan* had a bigger effect on the efficiency of transformation of cells with pBE3 than *clpX*^{hsdC} in the NM840 background (Tables 3.2 & 3.9). *E. coli* C *clpX*^{hsdC} derivatives (NK176) had a stronger barrier to the transfer of pBE3 than *E. coli* K-12 NM840 *clpX*^{hsdC} (NK180). However, the Amp^r transformants of both NK176 and NK180 that acquired pBE3 were restriction-proficient while no transformant of NM820 was obtained.

These results suggested that both NK180 and NK176 were different from HsdC$^+$ as well as from the original HsdC$^-$ cells and showed an intermediate phenotype (Table 3.9) similar to that of ClpX$^-$. These data suggest that the hybrid JR302 has a background different from both *E. coli* K-12 and *E. coli* C. Possibly, this difference depends on gene mutations that could enhance the effect of *clpX*^{hsdC} on the establishment of R-M systems. In this case the HsdC$^-$ phenotype is the result of more than one mutation. One chromosomal mutation occurred within *clpX* and others somewhere else. If the former is replaced for the wild type allele it is enough to restore HsdC$^+$.

3.5. Other known cytoplasmic proteases do not affect the establishment of the *Eco*KI and *Eco*AI systems.

Lon, ClpXP, ClpAP, ClpYQ, HflA and HflB are *E.coli* cytoplasmic proteases known so far. HflB is the only one found to be essential, but cells containing the *hflB29* mutation are viable and the CII protein of λ was found to be more stable in the mutant bacteria. *lon, clpA, clpY, clpQ, hflA* and *hflB* derivatives of NM840 were constructed

by P1 transduction and conjugated with JC9935 (F'101-201), JC9935 (F'101-202), JC9935 (F'101-301) and JC9935 (F'101-302) at a ratio of 1:10 (recipient : donor). The titre of recipients and transconjugants after 2.5h matings was scored. None of the mutants defective in a structural component of a protease showed an effect similar to that found in similar experiments for *clpP* and *clpX* cells. Perhaps if some of the proteases different from ClpXP are involved in the regulation of *hsd* genes expression their participation is very minor compared to that of ClpXP.

3.6. Discussion

Tight posttranscriptional regulation of activities of type IA and IB R-M systems enables genes encoding these systems to be transferred from one strain to another. The ClpXP protease has been shown to be involved in this regulation. Cells defective in the active protease die upon transfer of *hsd* genes following the establishment of an appropriate R-M system.

The protease is not involved in the establishment of the modification activity following the acquisition of the hsd_KMS genes; clp^+ and *clpX* cells show no difference in the rates with which they reach their maximal modification proficiency. A long period of time (12h or 10 generations) was shown to be necessary for modification to reach its peak activity, as assessed by the methylation of unmodified λ phages in a single round of infection. Probably, these data reflect the disappearance of unmodified targets on the host chromosome rather than the accumulation of the methyltransferase. During infection, phage DNA competes with the bacterial chromosome as a substrate for methylation. Eventually, when the chromosome becomes fully methylated, there is no longer any competition and the percentage of the phages modified during a single round of infection reaches its maximum. It is interesting, that Prakash-Cheng and Ryu (1993) detected restriction activity after about 13-15 generations, the time necessary to finish chromosome modification in my experiments. Perhaps, complete methylation of the chromosome is a signal for the expression of the restriction activity. For *Eco*AI the restriction activity was detected after about 6 generations (Kulik and Bickle, 1996), much earlier than that for *Eco*KI. *Eco*AI modifies ummethylated and hemimethylated DNA equally quickly (Suri and Bickle, 1985), while umodified targets sequences are

poor substrates for *Eco*KI (Dryden *et al.*, 1993). Therefore, it is logical to expect that the time necessary for *Eco*AI to convert an unmodified chromosome into a fully modified one is shorter than that for *Eco*KI.

If ClpXP does not influence the establishment of modification it must affect the expression of the restriction activity. The simplest explanation for the role of the protease is that ClpXP degrades HsdR and therefore delays the formation of the active nuclease. At the same time if neither HsdM nor HsdS is a substrate for proteolysis the methylase M_2S_1 would be active while the concentration of R-subunit would not be high enough to form $R_2M_2S_1$. Such a delay in the production of the restriction activity enables cells to survive the establishment of an R-M system.

The complex structures of type III and especially type I endonucleases allow regulation of their enzymatic activities based on the different affinities with which subunits bind to each other. Analysis of the *in vitro* assembly pathway of the *Eco*KI endonuclease revealed more than just two (M_2S_1 and $R_2M_2S_1$) complexes (Dryden *et al.*, 1997). However, only $R_2M_2S_1$ exhibited endonuclease activity, whereas interaction of R with M_1S_1 leads to a non-functional complex $R_1M_1S_1$. It was suggested that M_1S_1 at higher concentration than M_2S_1 might subvert R subunits and prevent their binding to M_2S_1. According to this idea, HsdC (ClpXP) may either degrade inactive complexes $R_1M_1S_1$, M_1S_1 and free R and M subunits or bind R to prevent its interaction with M_2S_1. In this case the role of ClpXP is to prevent the formation of the active nuclease. As an alternative, the protease might destroy $R_2M_2S_1$ complexes by removing and degrading the R-subunits.

According to the experiments described (Fig. 3.2 – 3.4 and Tables 3.2, 3.3) the *clpX* mutation has a stronger effect than *clpP* on the acquisition of *hsd* genes, as if ClpX helps cells to survive the establishment of the *Eco*AI or *Eco*KI systems despite the absence of the active ClpXP protease. Some preliminary experiments show that the over-expression of *clpX* at least partly suppresses a *clpP* mutation. ClpX possesses a chaperone activity, but it is not necessary for HsdR folding because the endonuclease activity is expressed in a *clpX* mutant. It might play an opposite role in the folding of HsdR, i.e. in shifting the protein from a functional conformation to an inactive one that in the absence of ClpP may lead to dissociation of ClpX and inactivated HsdR. Possibly, the protein molecules with the changed conformation are stably maintained in

the cytoplasm or even restored to the active form. If the interaction with ClpX results in the insolubilization of HsdR, the latter may aggregate. In any case ClpX could be a barrier to the binding of HsdR to M_2S_1, and this leads to a delay in expression of the endonuclease activity and the sequential establishment of methylation and restriction. Even if ClpX does not unfold HsdR, the ClpX-HsdR complex may be stable enough to prevent binding of HsdR to M_2S_1. In this case ClpX-mediated protection depends on the relative concentrations of ClpX and M_2S_1, the proteins competing for HsdR binding, and their affinity for HsdR. As an alternative, ClpX could remove HsdR from $R_2M_2S_1$ complexes and maintain a lower concentration of the active nuclease in cells.

The establishment of *Eco*AI is less sensitive to the absence of the ClpXP protease than that of *Eco*KI. This can be explained by the fact that the modification component of the *Eco*AI system is active on unmethylated target sequences (Suri and Bickle, 1985), whereas that of *Eco*KI has a very strong preference for hemimethylated DNA (Dryden *et al.*, 1993). Acquisition of hsd_A by P1 transduction does not require ClpXP for bacterial survival. Cells used for P1 transduction experiments are in a stationary phase when chromosome replication and cell division occur much more seldom than those in log-cultures. Constant cell volume when bacteria do not grow could help to accumulate the methylase despite a lower level of the protein synthesis in the cells than in exponentially growing bacteria. If the chromosome is not replicated the number of the target sequences does not increase with time. These two features of the stationary phase cells may make it easier for the methylase to complete the chromosome methylation.

Transfer of *hsd* genes by P1 transduction can be also influenced by the phage. The Dar protein encoded by P1 genome protects the donor DNA from restriction enzymes present in the recipient cells (Iida *et al.*, 1987). The mechanism of Dar action is unknown. If it temporarily blocks restriction but not modification the time interval could be enough for *Eco*AI (but not for *Eco*KI) to methylate the chromosome. Similar explanation might be applied to the experiments on the elimination of specificities by P1 transduction. If Dar blocks restriction for time necessary to dilute residual proteins, encoded by the genes eliminated, so much that no active endonuclease complex exists in the bacteria, then ClpXP would not affect the cell survival following the specificity elimination.

It is not clear whether ClpXP affects R-M systems constitutively or whether it is induced in response to a signal. The fact that *clp* mutants show a higher level of restriction than *clp*$^+$ bacteria suggests that the protease-dependent influence is not only a characteristic of the establishment; it is either constitutive or can be induced by some other processes in bacteria.

CHAPTER 4. REGULATION OF ENDONUCLEASE ACTIVITY PREVENTS BREAKAGE OF UNMODIFIED BACTERIAL CHROMOSOMES BY TYPE I RESTRICTION ENZYMES.

4.1. Introduction

It is very likely that the lethal effect of transfer of *hsd* genes that encode a new type I specificity to *clpP* or *clpX* bacteria is caused by breakage of unmodified chromosomal DNA by the endonuclease. This does not happen in clp^+ cells: the ClpXP protease is presumed to enable the establishment of modification and restriction so that the restriction activity appears only after the chromosomes are methylated. This idea is supported by the fact that bacteria take up to 15 generations after the acquisition of *hsd* genes to become restriction-proficient (Prakash-Cheng & Ryu, 1993; Kulik & Bickle, 1996), i.e. the restriction function is not active immediately in spite of the $hsdR^+M^+S^+$ genotype.

The acquisition of a new specificity is not the only situation in which a temporary loss of restriction proficiency has been detected. A well-documented example, referred to as restriction alleviation (RA), occurs in response to treatments that damage DNA (Day, 1977; Thoms & Wackernagel, 1984; Efimova *et al.*, 1988b). UV light, nalidixic acid, 2-aminopurine (2-AP) and 5-bromouracil (5-BU) have been shown to induce RA. It is possible that the temporary silence of restriction proficiency associated with the establishment of a new specificity is an example of RA. If this is so, ClpXP would be required for the alleviation of restriction in response to DNA damage.

Phage λ encode their own protein (Ral) which alleviates restriction of type IA systems. In the presence of Ral type IA systems methylate unmodified DNA, which is normally a very poor substrate for methylation, as effectively as hemimethylated. Some *E. coli* strains contain the Rac prophage in their chromosomes (Kaiser & Murray, 1979). The Rac prophage encodes Lar, a protein similar to Ral. Although some indirect data suggest that RA is not dependent on Ral (Efimova *et al.*, 1988a; Hiom & Sedgwick, 1992), *E. coli* K-12 C600 *rac* background was used in most of

the further experiments to identify Rac-independent regulation of restriction and modification activities of type I systems.

4.2. ClpXP is necessary for restriction alleviation.

Efficiency of plating of phage (e.o.p.), which is used in most cases to characterise restriction activity, reflects the probability with which a phage yields progeny on a given bacterial strain relative to a control strain. An e.o.p. of 1 means that there is no barrier for the phage to propagate on the respective strain either because of restriction-deficiency of the bacteria or because the phage has appropriate modification to protect against any resident restriction system. If unmodified phage are plated on restriction-proficient bacteria most of them are inactivated by the bacteria and therefore the e.o.p. is greatly reduced and reflects the probability with which the phages escape restriction. The inverse of e.o.p. measures restriction, or by what factor restriction activity prevents the production of an infective centre on restriction-proficient bacteria relative to that on restriction-deficient bacteria, where e.o.p. is assumed to be 1.

RA is a drop in restriction detected by the increased e.o.p. of unmodified λ on bacteria after they have been treated with UV light, nalidixic acid or 2-AP. RA was assessed for Clp^+ (NK301) and $ClpX^-$ (NK304) bacteria in response to each of the three treatments and was found to be ClpX dependent (Fig. 4.1). Similarly, a *clpP* mutant (NK303) treated with 2-AP was found to be deficient in RA (data not shown). RA for the *Eco*AI system in response to 2-AP also was shown to be dependent on ClpX (Fig. 4.2).

The results support the hypothesis that RA in response to agents that damage DNA, and the delayed detection of restriction activity following the acquisition of hsd^+ genes by an hsd^- recipient, are both the outcome of a common ClpXP-dependent process. RA therefore might be induced during the establishment of a new specificity and prevent cell death.

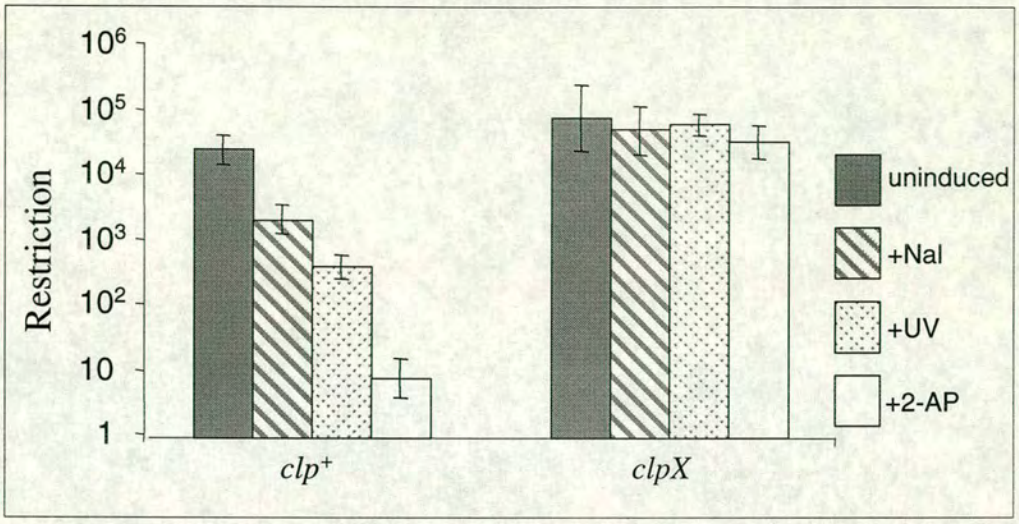


Fig. 4.1. Alleviation of the *Eco*KI restriction-proficiency in *clp*$^+$ (NK301) and *clpX* (NK304) bacteria.

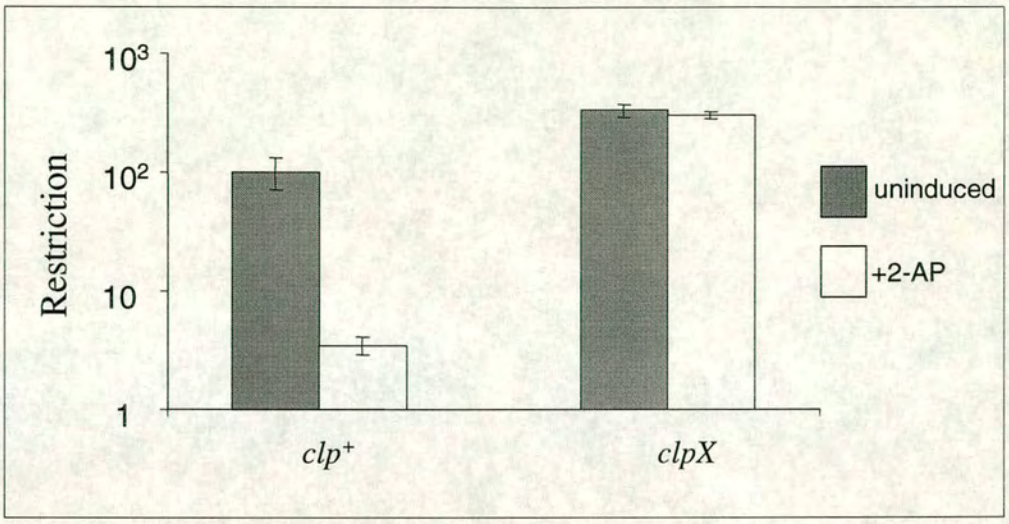


Fig. 4.2. 2-AP induced alleviation of the *Eco*AI restriction-proficiency in *clp*⁺ (NK354) and *clpX* (NK355) bacteria.

4.3. RA in cells with different dosage of the *hsd* genes

Gene dosage has been shown to affect the establishment of the *Eco*KI and *Eco*AI specificities (Suri & Bickle, 1985; Fuller-Pace *et al.*, 1985; Kelleher *et al.*, 1991). Transfer of *hsdM* and *hsdS* to recipient cells where *hsdR* was expressed from a multicopy plasmid was lethal to the bacteria in spite of their clp^+ allele. Does this mean that RA does not occur in cells over-expressing *hsdR*?

Clp^+ bacteria with a single copy of the hsd_KRMS genes on the chromosome (NK301) were transformed with plasmids of different copy number. The plasmids included one or more of the hsd_K genes and therefore conferred increased dosage of one, two or all three genes in the transformed cells. The cultures were grown in the presence and absence of 2-AP and assayed for restriction (Fig. 4.3). Restriction was considerably alleviated by the 2-AP treatment in all cases but in the bacteria with increased dosage of *hsdR*, the remaining level of restriction after the treatment was much higher than that for cells with a single copy of the gene (Fig. 4.3). The residual level of restriction might be of vital importance during the establishment of a new specificity. If restriction is not inactivated then it would act on unmodified chromosomal DNA.

4.4. Methyltransferase activity is not affected by either 2-AP or deficiency of ClpXP.

Restriction activity is greatly reduced by 2-AP in a ClpXP-dependent manner (Fig. 4.1 & 4.2). Is modification activity affected under the same conditions? λral^- (NM1090) and λral^+ (NM1094) phages were used as substrates for methylation to assay cells for modification activity. Ral enables phage to become effectively modified by their hosts. Unmethylated DNA, unlike hemimethylated, is a very poor substrate for methylation by *Eco*KI. In the presence of Ral *Eco*KI modifies unmethylated DNA effectively and therefore Ral^- phages are undermethylated by *Eco*KI (Loenen & Murray, 1986).

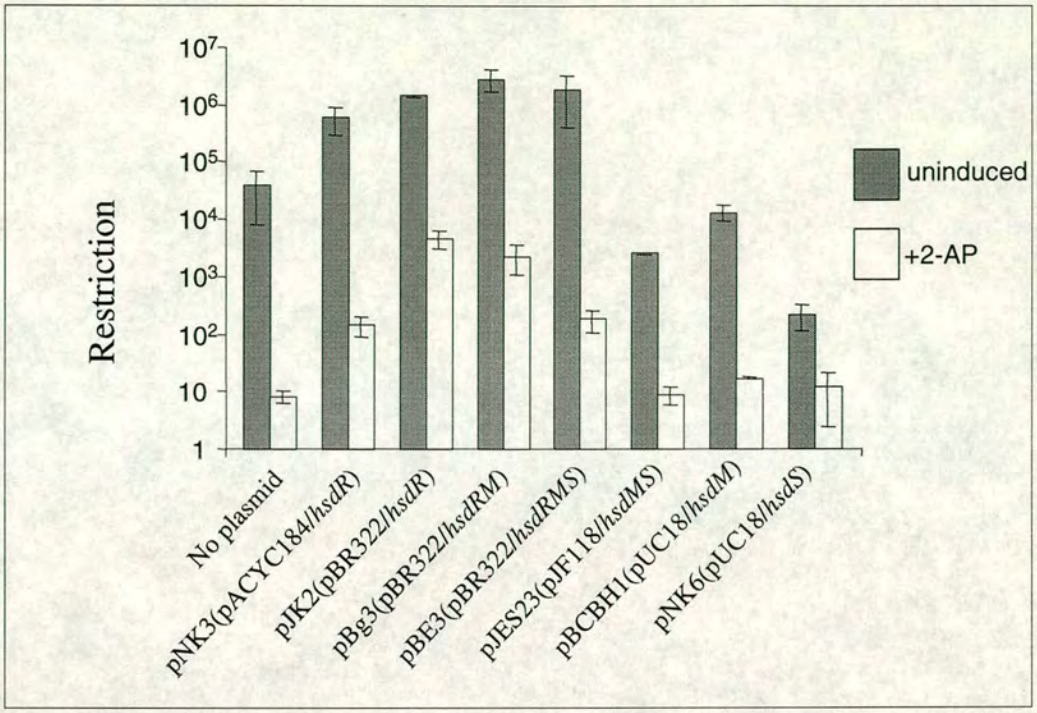


Fig. 4.3. Alleviation of the *Eco*KI restriction activity in *clp*$^+$ (NK301) bacteria with different dosage of the *hsd* genes.
Vector copy number:
pACYC184 ~8-10 per cell,
pBR322 ~20-25 per cell,
pUC18 ~200 per cell,
pJF118 is a pBR322-based vector with the p_{lac} promoter for the overexpression of cloned genes. pJES23 includes *hsdM*$^+$*S*$^+$ under a control of p_{mod} promoter and the p_{lac} promoter. The expression from the p_{lac} promoter was not induced with IPTG.

Table 4.1.

The *Eco*KI modification activity is not dependent on either ClpXP or 2-AP.

Host	2-AP	e.o.p. of progeny on restriction-proficient bacteria (NK301)	
		λ*ral*⁻ (NM1090)	λ*ral*⁺ (NM1094)
NK310	-	6.3×10^{-4}	0.74
	+	7.4×10^{-4}	0.86
NK310 *clpX*	-	5.0×10^{-4}	1.00
	+	7.6×10^{-4}	1.05

clp$^+$ and *clpX* restriction-deficient and modification-proficient ($r_K^-m_K^+$) bacteria (NK310 and NK325 respectively) were treated with 2-AP and infected with unmodified λ*ral*$^-$ and λ*ral*$^+$. Phage progenies produced after single rounds of infection were tested for modification. Neither *clpX* nor 2-AP treatment had an effect on the efficiency of modification (Table 4.1), consistent with the previous results on the independence of the establishment of modification on *clpX* (Chapter 3).

4.5. Transcription of the *hsdR*$_K$ gene is not changed during restriction alleviation.

RA is not the consequence of increased modification activity (see previous section) and therefore the drop in the level of restriction might reflect a decreased number of endonuclease (but not methylase) complexes per cell. This may be achieved either by a block in the synthesis of HsdR or by sequestering HsdR from its assembly with the methylase. Inhibition of the synthesis of different proteins occurs very often at the level of transcription of the appropriate gene. The activity of p_{res}, a promoter of the *hsdR*$_K$ gene, was assessed using transcriptional fusions of p_{res} with the coding sequence of the reporter gene *lacZ* (Loenen *et al.*, 1987). *clp*$^+$ (NK301) and *clpX* (NK304) bacteria were lysogenized with λ phages containing the fusions (λAD8 and λAD10, which have respectively 2kb and 0.3kb fragments of DNA upstream of the coding sequence of *hsdR* fused to *lacZ*) and with a control phage λTL25, the original vector without any insertion. The lysogens were grown in the presence and in the absence of 2-AP and assayed for β-galactosidase activity and restriction. The activity of β-galactosidase was not affected either by *clpX* or by 2-AP (Table 4.2) while RA was dependent on both *clpX* and 2-AP (data not shown). The data suggest that the regulation of endonuclease activity during RA occurs posttranscriptionally.

Table 4.2. p_{res} activity under RA-inducing conditions.

Bacteria	$p_{res\text{-}lacZ}$ fusion	β-galactosidase activity, MU	
		-2-AP	+2-AP
clp$^+$:			
- NK301(λTL25)	-	76.6 ± 9.0	78.5 ± 7.6
- NK301(λAD8)	+	783.6 ± 21.3	687.1 ± 46.2
- NK301(λAD10)	+	841.8 ± 45.7	642.3 ± 13.7
clpX:			
- NK304(λTL25)	-	77.02 ± 13.8	67.5 ± 12.1
- NK304(λAD8)	+	637.1 ± 28.8	600.0 ± 50.8
- NK304(λAD10)	+	769.4 ± 74.1	627.8 ± 65.7

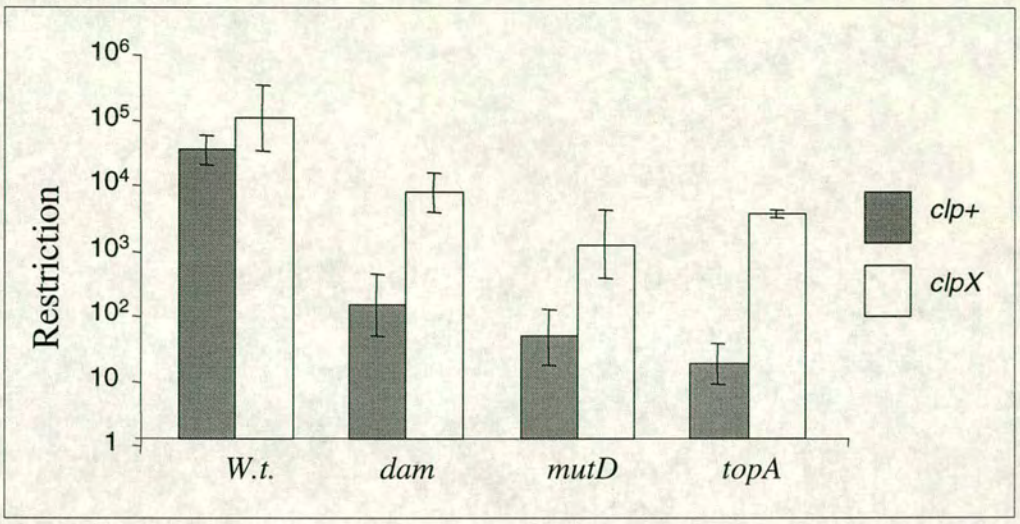


Fig. 4.4. Restriction of unmodified λ by *dam* (NK302), *mutD* (NK326), *topA* (RS2) strains and their *clpX* derivatives (NK315, NK327 and NK329).

4.6. "Constitutive" restriction alleviation

2-AP is known to stimulate mismatches during chromosome replication (Ronen, 1980). Are mismatches relevant to induction of RA by 2-AP or is RA a result of an unknown effect of 2-AP on bacterial cells? Some mutations, which affect either the fidelity of replication or repair of mismatches, lead to an increased level of mismatches in bacteria. Mutations in any of the *mut* genes (*mutH*, *mutL* or *mutS*) encoding components of the mismatch repair system have been shown to relieve restriction about 5 fold (Efimova *et al.*, 1988b). The fidelity of replication can be abolished by a *mutD* (*dnaQ*) mutation (Echols *et al.*, 1983). *mutH::kan*, *mutD::*Tn*10* and *mutH::kan mutD::*Tn*10* derivatives of NK301 were constructed and assayed for restriction. In the MutH$^-$ bacteria restriction of unmodified λ was derepressed 4-fold, in MutD$^-$ cells 60-fold, and in the double mutant restriction was alleviated 400-fold. In the last case the level of restriction (10 or e.o.p. 10^{-1}) was similar to that in NK301 treated with 2-AP and probably reflects a similar level of mismatches in the bacteria. It is logical to expect that the number of mismatches in the double *mutH::kan mutD::*Tn*10* mutant is higher than in either of the singles and therefore according to the data the higher the level of mismatches the greater is the level of RA. The results suggest that mismatches *per se* are the agent inducing RA. Because the number of mismatches per cell in the *mut* mutants is permanently increased the RA in these bacteria is "constitutive".

Restriction is alleviated in *dam* strains (Efimova *et al.*, 1988a). The Dam-methylation is used by the MutHSL system to identify the parental DNA strand during mismatch repair and in *dam* mutants mismatch repair leads to double-strand breaks (DSBs) (Wang & Smith, 1986) followed by SOS response and RecA-dependent repair. SOS response is also switched on in UV-treated cells which have been shown to have RA. If SOS response causes RA then a lower level of restriction in *dam* cells can be considered as another case of "constitutive" RA.

topA mutants has been found to restrict unmodified λ less well than *topA*$^+$ bacteria (G.P.Davies & N.E.Murray, pers.comm.) as if they also show "constitutive" RA. Mutants deficient in topoisomerase I, like wild-type cells treated with nalidixic acid, have problems in DNA replication. When topological problems make replication

forks stall DSBs may occur (Michel *et al.*, 1997) and induce RecA-dependent DNA repair.

If poor restriction described for *mut*, *dam* and *topA* mutants is an example of constitutively induced RA then a mutation in *clpP* or *clpX* should restore restriction. Consistent with this prediction, the efficiency of restriction was enhanced by approximately one hundred-fold in the absence of ClpXP protease (Fig. 4.4).

4.7. RA in response to 2-AP is not dependent on SOS-induced DNA repair.

It has been found that UV-RA and Nal-RA do not occur in *recA* and *lexA*(Ind$^-$) bacteria (Day, 1977; Thoms & Wackernagel, 1984). *recBC* and *recF* mutants also are deficient in RA in response to UV light and functional *recBC* is necessary for Nal-RA (Thoms & Wackernagel, 1982, 1984; Kelleher & Raleigh, 1994). Mutations in *umuD* or *umuC* lead to a reduction of RA although bacteria still respond to UV treatment by weaker restriction (Hiom & Sedgwick, 1992). In all these mutants, either the SOS response is blocked completely (e.g. *recA* and *lexA*(Ind$^-$) cells) or some DNA repair pathways normally activated during SOS are not functional (e.g. *recBC, recF* and *umuDC* bacteria). On the basis of these earlier observations UV-RA and Nal-RA appear to be a function of SOS response.

RA in response to 2-AP has been shown to be independent of *recA* and *lexA* (Efimova *et al.*, 1988b). Do other mutations that abolish UV-RA have an effect on RA upon 2-AP treatment? *recA, recBC, recD* and *umuDC* derivatives of NK301 were made and, together with *clpP* and *clpX* bacteria, were treated with 2-AP and assayed for restriction. Only a ClpXP deficiency led to a block of RA, all the other mutants behaved like the original strain, NK301, and restricted poorly in the presence of 2-AP (Table 4.3). The observation that neither UV light nor 2-AP induce RA in ClpXP-deficient cells implies the existence of a common step for both pathways. The different effects of mutations in *recA*, *lexA*, *recBC*, and *umuDC* on UV-RA and 2-AP-RA, however, suggest that some stages of the UV-induced RA pathway are different from those for 2-AP and require functional pathways for DNA repair.

Table 4.3. Comparison of RA induced by UV light and 2-AP [*].

Bacteria	UV-induced RA	2-AP-induced RA
NK301	~ 10^4 [(a,b)]	1.0×10^4
NK303 (*clpP*)	n.t.	1.1
NK304 (*clpX*)	1.0	0.9
NK305 (*recD*)	n.t.	5.8×10^3
NK306 (*recBC*)	~ 1 [(c)]	9.2×10^3
NK307 (*umuDC*)	~ 10^2 [(a)]	3.8×10^4
NK308 (*recA*)	1.2×10^1 [(b)]/ ~1.0 [(d)]	1.0×10^4

[*] The data accompanied by the references are obtained for mutations in different backgrounds. n.t. – not tested.

[a] Hiom & Sedgwick, 1992.

[b] Kelleher & Raleigh, 1994.

[c] Thoms & Wackernagel, 1982

[d] Day, 1977.

4.8. Prolonged 2-AP treatment is lethal for ClpXP-deficient cells; the lethality is caused by functional *Eco*KI.

An attempt to induce RA in the *clp* mutants by prolonged treatment with 2-AP led to an unexpected observation; after 4-5h incubation in 2-AP the viability of *clpP* and *clpX* cells dropped so much that it became impossible to use the cultures to assay for restriction (No confluent bacterial lawn was obtained). Examination of bacteria under the light microscope revealed that ClpXP-deficient cells became filamentous after 3-4h incubation in the presence of 2-AP. The filamentous cells remained mobile. They increased in length during the next 2h and then lost their mobility, correlating with cell death. Similar experiments for *recA, recBC, recD* and *umuDC* derivatives did not reveal any effect of 2-AP on their survival.

Filamentation is a phenotype characteristic of the SOS response. 2-AP does not normally activate the SOS response but in the absence of ClpXP it could induce a chain of events that leads to DNA damage. This DNA damage would require RecA for repair and induction of the SOS response. This idea is supported by the fact that *clpX recA* double mutants (NK323) are supersensitive to 2-AP and do not survive low concentrations (40 μg/ml) of 2-AP in the medium (Table 4.4). In contrast, clp^+ *recA* cells (NK308) are not sensitive to 2-AP and show RA in response to treatment with 2-AP (Table 4.3). It is interesting that about 20% of *clpX recA* cells treated with 2-AP are filamentous in spite of their deficiency in RecA, which is necessary for induction of filamentous growth as the function of the SOS response.

Is ClpXP needed in the presence of 2-AP to prevent DNA damage by the resident restriction endonuclease? If so, then the inactivation of *Eco*KI would suppress the lethal effect of 2-AP for the *clp* mutants. *Eco*KI was inactivated either by introducing a point mutation in *hsdR* that abolishes restriction but not modification activity of the enzyme, or by deleting all the *hsd* genes. Both mutations restored the resistance of the ClpXP-deficient cells to 2-AP (Table 4.4). Similarly, the hypersensitivity of the *clpX recA* strain was relieved by inactivation of *Eco*KI (Table 4.4).

The data suggest that in the presence of 2-AP *Eco*KI becomes potentially harmful for a host cell and that ClpXP is necessary to prevent *Eco*KI from causing cell death. In the absence of ClpXP and in the presence of the functional *Eco*KI endonuclease the

SOS response is activated. These observations imply that the *Eco*KI endonuclease activity damages host DNA that contains unmodified target sequences and therefore becomes a substrate for restriction by *Eco*KI. This harmful effect of *Eco*KI occurs only in the presence of 2-AP. How does 2-AP induce the appearance of unmodified targets in the *E. coli* chromosome? 2-AP is known to stimulate transitions during DNA replication. Such point mutations can convert some nucleotide sequences to targets for *Eco*KI. Originally, these sequences have never been recognised as targets because they were different from the target sequence by a single base pair. 2-AP induced substitution of this base pair with another one would result in the appearance of a new recognition site for *Eco*KI. Such newly synthesised target sequences in a bacterial chromosome would not be methylated and would make the chromosome a substrate for cleavage by *Eco*KI. However, DNA breakage does not occur in the presence of functional ClpXP. The fact that *recA clp*$^+$ bacteria are not sensitive to 2-AP supports the idea that double-strand breaks, which would be lethal for *recA* mutants, do not occur during RA, rather chromosome breakage takes place in Clp$^-$ cells which cannot induce RA.

4.9. RA induced by 2-AP is associated with a deficiency of HsdR.

A drop of restriction in response to 2-AP, UV or nalidixic acid might be caused by a deficiency of HsdR in treated cells. The lower the concentration of HsdR the lower would be the concentration of the endonuclease complexes $R_2M_2S_1$ in cells and the lower the probability of restricting unmodified DNA. To test this idea, the HsdR and HsdM subunits were monitored by Western blots following the addition of 2-AP to both *clp*$^+$ (NK301) and *clpX* (NK304) bacteria (Fig. 4.5). After a lag of 20 min, a reduction in the amount of HsdR per constant volume of culture was detected in *clp*$^+$ cells in response to 2-AP. At the same time, the cell growth was accompanied by accumulation of HsdM. Under the same conditions, *clpX* cells behaved like untreated bacteria and accumulated both HsdR and HsdM (Fig. 4.5).

Table 4.4. Bacterial growth upon prolonged treatment (4-5h) with 2-AP.

Bacteria	Concentration of 2-AP in LB		
	0 μg/ml	40 μg/ml	400 μg/ml
NK301 (*hsd*$^+$ *clp*$^+$)	normal	normal	normal
NK304 (*hsd*$^+$ *clpX*)	normal	~ 20% filaments	100% filaments, cell death
NK323 (*hsd*$^+$ *clpX recA*)	normal	~ 20% filaments, cell death	~ 20% filaments, cell death
NK310 (*hsdR clp*$^+$)	normal	normal	normal
NK325 (*hsdR clpX*)	normal	normal	normal
NK311 (*Δhsd clp*$^+$)	normal	normal	normal
NK312 (*Δhsd clpX*)	normal	normal	normal
NK324 (*Δhsd clpX recA*)	normal	normal	normal

The conclusion about cell death relied on the loss of mobility of cells.

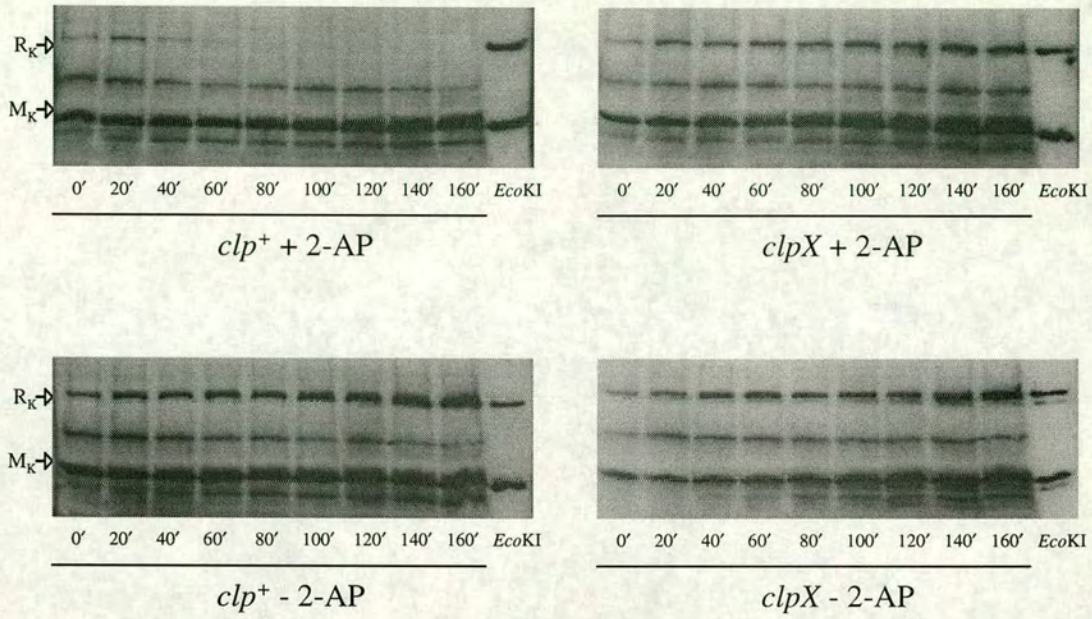


Fig. 4.5. Assays for HsdR and HsdM polypeptides following treatmentwith 2-AP. 2-AP was added to mid-logarithmic cultures at the point 0′ and 0.5 ml aliquots were taken at appropriate intervals and analysed in Western blot experiments. *Eco*KI polyclonal antibody, used in these Western blots, fails to detect HsdS, but detects HsdR and HsdM and some other *E. coli* proteins.

4.10. 2-AP induces ClpXP-dependent degradation of HsdR.

The very low concentration of HsdR in *clp*$^+$ cells treated with 2-AP can be explained in two ways; either HsdR is unstable in the presence of ClpXP or HsdR is unstable because of another protease but ClpXP is necessary to stop the synthesis of HsdR in 2-AP treated cells.

The stability of HsdR was assayed in *clp*$^+$ and *clpX* cells treated with 2-AP. When *hsdR* was present as a single copy per cell, the amount of HsdR was insufficient to generate a signal that would readily distinguish HsdR from other proteins on SDS polyacrylamide gels. The signal was increased by using *hsdR* cloned in pACYC184, a low copy number vector. *clp*$^+$ *hsd*$^+$ (NK301) and *clpX hsd*$^+$ (NK304) bacteria transformed with the *hsdR*$^+$ plasmid (pNK3) were treated for 90 min with 2-AP to establish RA and pulse-labelled with ^{35}S-methionine. HsdR was unstable in *clp*$^+$ but not *clpX* cells (Fig. 4.6). In the absence of 2-AP HsdR was stable in both strains. The ClpXP-dependent degradation of HsdR is inducible since the polypeptide is stable in untreated cells and becomes a substrate for proteolysis in the presence of 2-AP.

4.11. Functional *Eco*KI is necessary for the loss of HsdR during RA.

The ClpXP-dependent degradation of HsdR is an inducible process, i.e. HsdR becomes a substrate for proteolysis only under special conditions. Is active *Eco*KI necessary to generate the signal that leads to ClpXP-dependent degradation of HsdR? If it is not then does ClpXP attack HsdR on its own or as a part of a complex with HsdM and HsdS? To answer these questions two mutants were tested for 2-AP induced depletion of HsdR. One had a missense mutation in *hsdR* (NK351) that retained the ability of the $R_2M_2S_1$ complex to bind DNA (Davies *et al.*, 1998) but inactivated both the ATP-dependent translocation activity and endonuclease activity (Davies *et al.*, 1998, 1999b). The other mutant (NK352) had a wild-type *hsdR* gene but *hsdM* and *hsdS* were deleted so that HsdR could not form an *Eco*KI complex.

HsdR was not depleted in either mutant in response to 2-AP (Fig. 4.7a). This finding implies that a functional endonuclease is required for induction of the pathway that leads to degradation of HsdR.

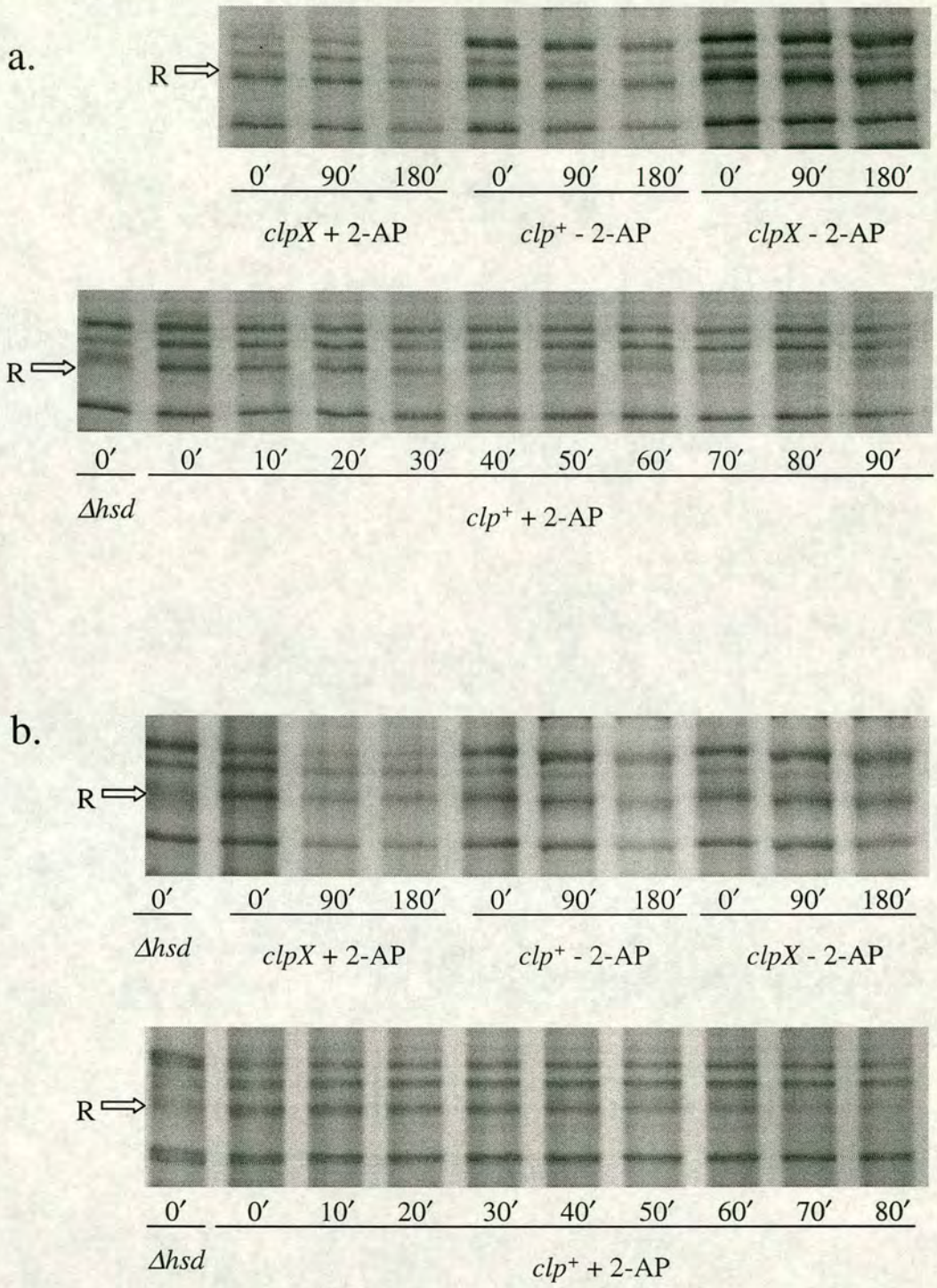


Fig. 4.6. The stability of HsdR *in vivo*. Labelled polypeptides separated by electrophoresis through SDS-polyacrylamide gels (6%) were detected by autoradiography. An extract from a strain lacking HsdR (NK311/pACYC184) was used to identify HsdR in the other strains. Samples from *clp*$^+$and *clpX* bacteria containing pNK3 were taken at time intervals indicated after pulse labelling. (a) and (b) are two independent experiments.

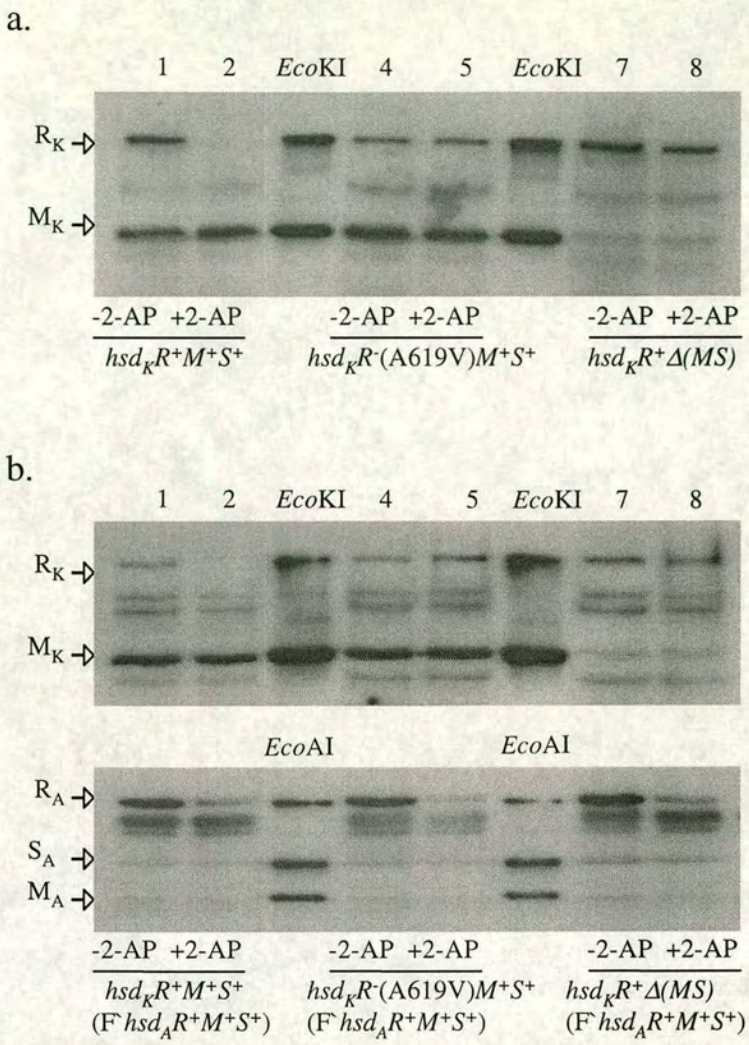


Fig. 4.7. Functional *Eco*KI is necessary for degradation of $HsdR_K$. Hsd subunits were monitored by Western blot following treatment with 2-AP, using antibodies raised against the relevant R-M complex. (a) Degradation of HsdR is prevented by a missense mutation in *hsdR* (track 5) or by the absence of HsdM and HsdS (track 8). (b) The presence of functional *Eco*AI has no effect on the degradation of the HsdR subunit of *Eco*KI (*Upper*), even though the HsdR subunit of *Eco*AI itself is degraded (*Lower*, tracks 2,5 and 8).

If the products of restriction by a type I enzyme are the stimulus for RA, the endonuclease activity of one R-M system should induce RA for a different system. The degradation of the HsdR polypeptide of the inactive type IA system, *Eco*KI, was examined under conditions shown to induce RA for *Eco*AI. *Eco*AI is a type IB system for which RA is regulated in a ClpXP-dependent manner (Fig. 4.2).

F'$hsd_AR^+M^+S^+$ (F'101-301) was transferred to the three strains used in the previous experiment (Fig. 4.7a); $hsd_KR^+M^+S^+$, $hsd_KR^-M^+S^+$ and $hsd_KR^+\Delta(MS)$. The transconjugants, both treated and untreated with 2-AP, were assayed for *Eco*AI- and *Eco*KI-dependent restriction *in vivo*, and for the presence of HsdR polypeptides. 2-AP caused RA of functional R-M systems, and HsdR from any restriction-proficient complex was lost (Fig. 4.7b). However, for the non-functional *Eco*KI complex $HsdR_K$ remained even when $HsdR_A$ of functional *Eco*AI was degraded.

Double-strand breaks (DSBs) are known to be the products of restriction by type I R-M systems. If DSBs were the signal for RA then breaks generated by *Eco*AI would have induced degradation of $HsdR_K$. The results, however, do not support this idea. In addition, if DSBs occurred during RA then *recA* bacteria would have had a problem to survive 2-AP because RecA is necessary for DSBs repair. $RecA^-$ cells are not sensitive to 2-AP and show RA (see 4.7 & 4.8). This implies that DSBs are not produced during RA, rather they are prevented by ClpXP.

If 2-AP stimulates the appearance of unmodified target sites in the chromosome (see 4.8) then *Eco*KI should recognise them and initiate the restriction pathway leading to DNA cleavage. Let us compare the restriction pathways of wild-type *Eco*KI, in which HsdR is degraded in the presence of 2-AP, with the enzyme in an *hsdR* mutant (NK351) in which HsdR is non-degradable. They both form $R_2M_2S_1$ complexes that recognise DNA in a site-specific manner but the former will translocate and cleave the DNA whereas the latter will not. The cleavage step does not happen in either situation and therefore the known difference between the complexes is their ability to translocate DNA. These results provoke the suggestion that when the endonuclease translocates chromosomal DNA, HsdR is recognised by ClpXP, removed from the complex and degraded by the protease. This idea also would explain why functional *Eco*AI had no effect on degradation of HsdR of non-functional *Eco*KI.

4.12. Discussion

Two phenomena reported for type I R-M systems implied the existence of a regulatory pathway modulating at least one of the two activities of these systems. One was the puzzling fact that bacteria survived the acquisition of genes specifying a new type one R-M system (Prakash-Cheng & Ryu, 1993; O'Neill *et al.*, 1997). When *hsd* genes are expressed in a naive cell, the first few molecules of nuclease are expected to appear before the methylation of the chromosome is complete and therefore the bacterium should die as the result of multiple cleavages of the chromosome by the nuclease. However, experimental data do not confirm this reasoning; the transfer of a new type I specificity does not have any effect on survival of recipient bacteria.

The other evidence for regulation of type I systems is based on observations that restriction is dependent on the physiological state of cells. Under some conditions bacteria show a temporal loss of restriction proficiency in spite of their $hsdR^+M^+S^+$ genotype. Such alleviation of restriction is induced in response to UV irradiation, growth in the presence of sub-lethal concentrations of nalidixic acid, 5-BU or 2-AP (Bertani & Weigle, 1953; Thoms & Wackernagel, 1984; Efimova *et al.*, 1988b). Removal of the inducing agent leads to a gradual recovery of restriction.

Neither the acquisition of a new specificity nor prolonged treatment with 2-AP affects the survival of clp^+ bacteria but both cause cell death of mutants deficient in ClpXP protease. The dependence of these processes on the protease implies a common ClpXP-dependent regulatory pathway which is necessary to survive both the acquisition of an hsd^+ allele and growth in the presence of 2-AP.

Cell death following the treatment of *clpX* and *clpP* bacteria with 2-AP is accompanied by the formation of filaments, a characteristic of the induction of the SOS response. SOS is activated in response to DNA damage and in this case the evidence indicates that the damage is caused by active *Eco*KI; a mutation which blocks restriction activity prevents filamentous growth and cell death of *clpX* bacteria in the presence of 2-AP (see 4.8). How do clp^+ bacteria survive 2-AP, even without SOS induction? Clp^+ cells respond to 2-AP treatment by the degradation of HsdR and consequent inactivation of the endonuclease activity that becomes dangerous for

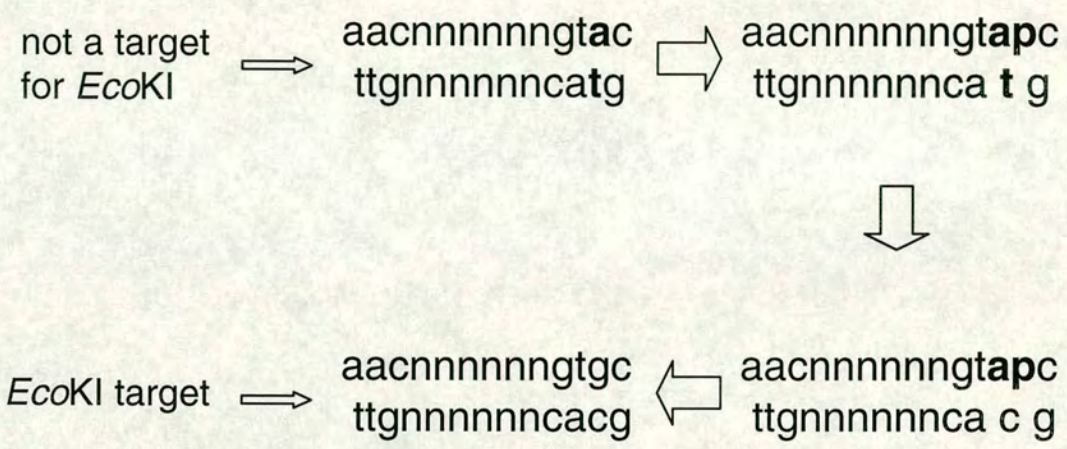
bacteria growing in the presence of 2-AP. Because this degradation is ClpXP-dependent, *clpX* and *clpP* mutants cannot remove HsdR and consequently they retain normal levels of endonuclease.

Why do 2-AP, UV irradiation and nalidixic acid induce cell death of Clp$^-$ bacteria? The *Eco*KI-dependent induction of SOS in ClpXP-deficient cells in response to 2-AP treatment suggests that the chromosomal DNA becomes a substrate for restriction by *Eco*KI. *Eco*KI cleaves only DNA with unmodified targets. 2-AP is known to induce transitions during DNA replication, mostly from A-T to G-C (Osborn *et al.*, 1967; Freeze, 1968). This could lead to the appearance of unmodified target sites in the chromosome *de novo*. There are sequences in the *E. coli* chromosome which are different from the *Eco*KI recognition sequence by a single base pair. If a 2-AP-induced mismatch eliminates the difference then a new target for *Eco*KI will appear (Fig. 4.8a). A similar mechanism could also operate for 5-BU.

*Eco*KI recognises AAC(N_6)GTGC as a target sequence and there are 596 such sequences in the *E. coli* K-12 chromosome. AA**T**(N_6)GTGC, AAC(N_6)**A**TGC, AAC(N_6)GT**A**C, and AAC(N_6)GTG**T** are different from the target by a single base shown in bold and if a substitution of **A-T** with G-C occurs it will convert any of these sequences to an *Eco*KI target. On the basis of probability of random sequences, one might expect about 600 such original sequences each, defined by 7 bp as well as the target sequence, per *E. coli* chromosome. Therefore there are about 2400 "potential targets" per chromosome. 2-AP, when it competes with adenine for incorporation during bacterial DNA replication, appears in the synthesised product as one base per 250-270 adenine bases (Gottschling & Freeze, 1961; Rogan & Bessman, 1970). This rate of 2-AP incorporation might result in the generation of up to 10 new *Eco*KI target sequences per chromosome per single round of replication.

In addition, 2-AP might stimulate the generation of unmodified target sequences by blocking methylation (Fig. 4.8b). When 2-AP is incorporated in an *Eco*KI target instead of adenine, the target for methylation, the R-M enzyme still recognises the sequence as a target (D.Dryden, pers.comm.) but cannot methylate the 2-AP residue and the DNA stays hemimethylated. The next round of replication, unavoidably, will result in an unmethylated target sequence.

a.



b.

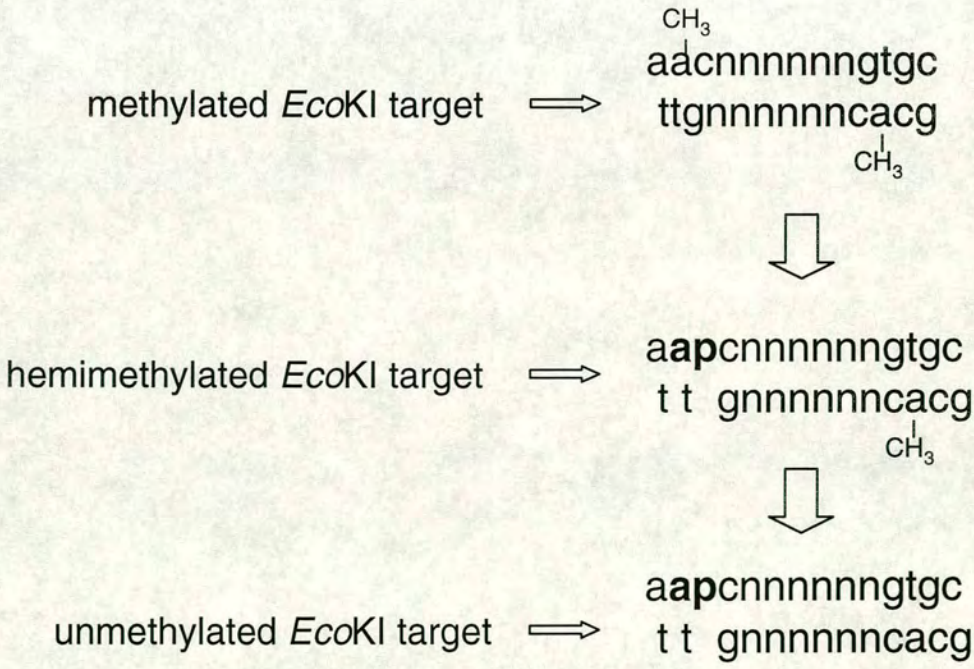


Fig. 4.8. Replication in the presence of 2-AP generates unmodified targets for *Eco*KI.

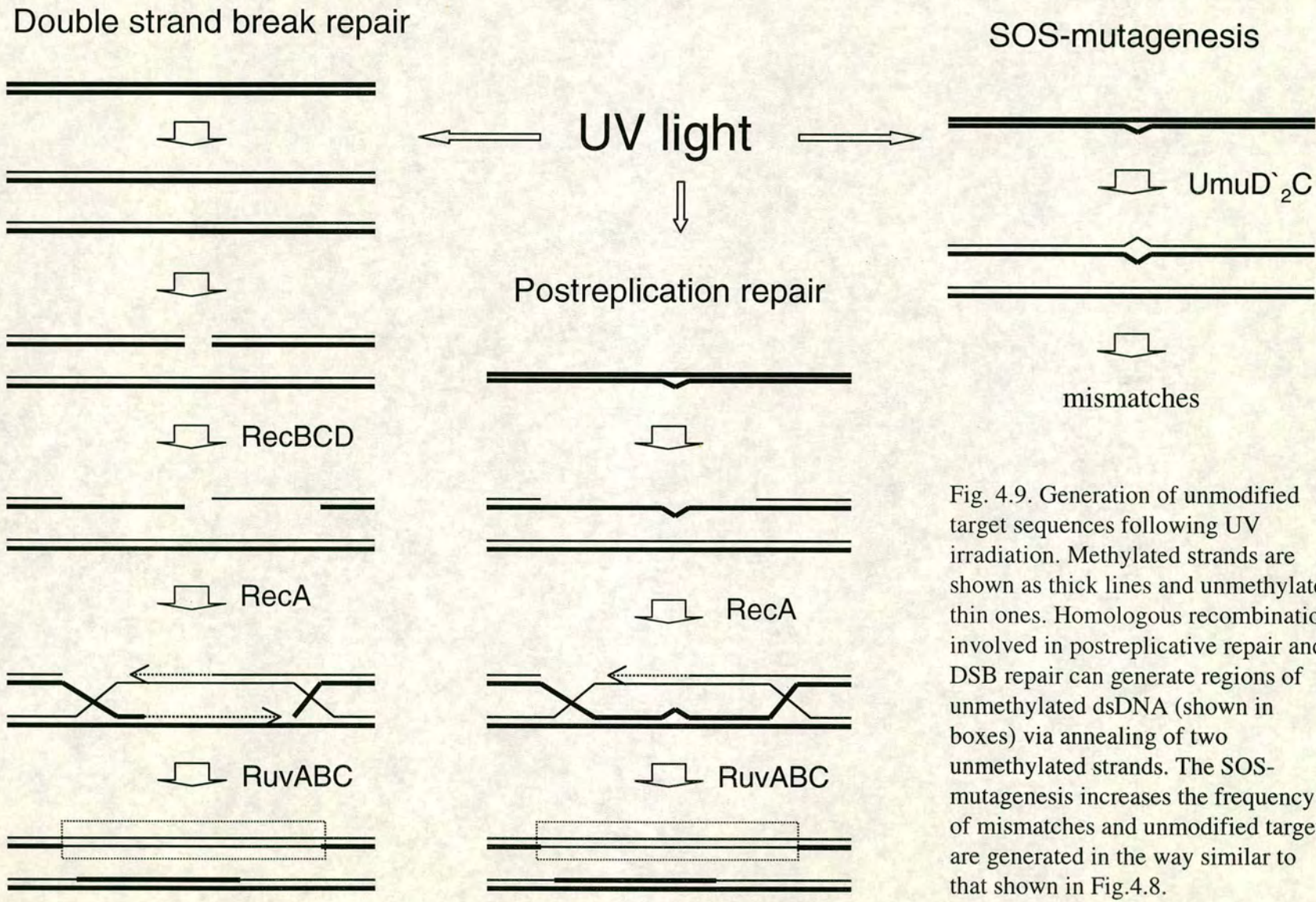


Fig. 4.9. Generation of unmodified target sequences following UV irradiation. Methylated strands are shown as thick lines and unmethylated as thin ones. Homologous recombination involved in postreplicative repair and DSB repair can generate regions of unmethylated dsDNA (shown in boxes) via annealing of two unmethylated strands. The SOS-mutagenesis increases the frequency of mismatches and unmodified targets are generated in the way similar to that shown in Fig.4.8.

If the appearance of unmodified targets leads to RA then how can unmodified targets be generated by UV light or treatment with nalidixic acid? When irradiated with UV, cells activate the SOS response including the SOS-mutagenesis pathways. One of the pathways is the SOS-aided translesion synthesis dependent on UmuD′$_2$C – RecA and producing mainly base changes (Miller & Low, 1984). Generation of new targets by this mechanism is similar to that for 2-AP and 5-BU, mismatches convert defined sequences to target recognition sites. Involvement of UmuD′$_2$C in RA is supported by the fact that *umuD* and *umuC* mutants showed a 100-fold lower level of UV-induced RA than *umu*$^+$ cells (Hiom & Sedgwick, 1992).

The other pathway for the generation of new target sequences may be activated in response to both UV irradiation and nalidixic acid. In both cases it would be based on homologous recombination as a process involved in the repair of DSBs. Nalidixic acid inhibits DNA gyrase (Gellert *et al.*, 1977; Sugino *et al.*, 1977) and causes DSBs in DNA (Snyder & Drlica, 1979; Drlica *et al.*, 1980) that induce the SOS response. Repair of DSBs and postreplication repair, both of which occur after UV irradiation, rely on homologous recombination. If homologous recombination involves two segments of hemimethylated DNA, the annealing of unmethylated strands or DNA synthesis may generate a localised region of unmethylated DNA (Fig. 4.9). Homologous recombination is strongly dependent on RecBCD and RecA. Because recombination does not occur in *recA* mutants and is greatly reduced in *recB recC* cells, the mechanism of generation of unmodified targets is blocked and therefore the mutants are deficient in UV-induced RA (Day, 1977; Thoms & Wackernagel, 1982, 1984; Kelleher & Raleigh, 1994). The UmuD′$_2$C-dependent pathway may be blocked in *recB* and *recC* mutants treated with nalidixic acid because RecBC is necessary to activate the SOS response after the treatment with nalidixic acid (Gudas & Pardee, 1976) and consequent derepression of *umuDC*. It remains unclear why *recB* and *recC* bacteria are deficient in UV-RA (Dharmalingam & Goldberg, 1980; Thoms & Wackernagel, 1982; Kelleher & Raleigh). The induction of the SOS response after UV irradiation does not require RecBC (Bockrath & Hanawalt, 1980; McPartland *et al.*, 1980) and occurs in a RecF-dependent manner (McPartland *et al.*, 1980; Karu & Belk, 1982). A mutation in *recF* blocks UV-RA (Thoms & Wackernagel, 1984; Kelleher & Raleigh, 1994). DNA replication of the damaged template is known to be

required to generate the SOS-inducing signal (Salles & Defais, 1984). The finding that *recF* bacteria are deficient in the resumption of replication at DNA replication forks after UV irradiation (Courcelle *et al.*, 1997) may explain the lack of RA in *recF* bacteria.

The LexA (Ind$^-$) phenotype is accompanied by a deficiency in UV-RA and Nal-RA (Thoms & Wackernagel, 1982, 1984) since the cells are unable to derepress transcription of *recA* and *umuDC,* which are involved in the generation of new target sequences.

The finding that DNA damage *per se* and DNA repair proteins are required to induce UV-RA is consistent with a pathway that leads to the generation of unmodified targets. Constitutive expression of *recA* in the absence of DNA damage does not cause RA (Thoms & Wackernagel, 1984; Hiom & Sedgwick, 1992) because there is no DNA substrate for homologous recombination or UmuD′$_2$C activity to initiate the formation of unmodified target sequences.

RA in response to 2-AP is different from UV-RA and occurs in *recA, recBCD, lexA* (Ind$^-$) and *umuDC* mutants. This is not surprising because the mechanism of appearance of new *Eco*KI targets induced by 2-AP differs from that for UV-RA and does not require the products of any of these genes. The idea that mismatches are a signal for RA is supported by the fact that an increased level of naturally occurring mismatches in mutants with either disturbed mismatch repair system (*mutH, mutL* and *mutS*) or with abolished fidelity of replication (*mutD*) leads to a drop in restriction (Efimova *et al.*, 1988a; see 4.6). Inactivation of both systems in the *mutH mutD* double mutant decreases restriction to the very low level found for 2-AP treated cells.

Similarly, RA induced by UV and nalidixic acid can be mimicked by *dam* and *topA* mutations respectively. In the absence of Dam-methylation, which is used for identification of the parental strand during mismatch repair by the MutHLS system (Wagner & Meselson, 1976; Pukkila *et al.*, 1983), the MutH endonuclease activity generates DSBs (Wang & Smith, 1986), one of the DNA products generated following UV irradiation. DNA replication in *topA* mutants, as well as in cells where GyrA is blocked by nalidixic acid, should encounter topological problems; this could lead to DSBs.

RA develops gradually after UV irradiation, or upon adding 2-AP, and achieves its maximum after 2-3h for UV and 1-1.5h for 2-AP (Efimova *et al*, 1988b; Kelleher & Raleigh, 1994). This time lag may be necessary for the generation of unmodified target sequences and might be influenced by growth conditions. Aeration affects the growth rate and therefore the rate of initiation of replication. Increased DNA synthesis leads to more 2-AP incorporation and consequently to the generation of more unmodified target sequences.

RA is abolished by the presence of chloramphenicol in growth media (Efimova *et al*, 1988b; Kelleher & Raleigh, 1994), implicating a requirement for protein synthesis for RA. It is known that protein synthesis is obligatory for the initiation of chromosome replication (Ward & Glaser, 1969) and this would explain the dependence of 2-AP-induced RA on chloramphenicol. For UV-RA, protein synthesis is necessary to produce UmuD, UmuC, RecA and, possibly, some other SOS-induced polypeptides involved in the generation of unmodified targets.

Unmodified targets on the chromosomes of $hsdR^+M^+S^+$ cells is a common problem occuring not only after treatments with UV, 2-AP, 5-BU or nalidixic acid, but also during the establishment of genes conferring a new specificity. The solution to this problem requires ClpXP to degrade HsdR. In *clpX* and *clpP* cells HsdR cannot be degraded and hsd^+ bacteria die after 2-AP treatment (see 4.8) while hsd^- cells die after the acquisition of an hsd^+ allele (see Chapter 3). The establishment of a new type I specificity is lethal even for Clp^+ bacteria if *hsdR* is expressed from a multicopy plasmid prior to the transfer of $hsdM^+S^+$ (Suri & Bickle, 1985; Fuller-Pace *et al.*, 1985; Kelleher *et al.*, 1992). The experiments on RA, when *hsd* genes are expressed from multicopy plasmids (Fig. 4.3), show that if the dosage of *hsdR* is increased then restriction cannot be alleviated to a level as low as that obtained for a single copy of *hsdR*. Perhaps there is not enough ClpXP protease to inactivate sufficient HsdR for the cell to survive the establishment of a new specificity when *hsdR* is expressed from a multicopy plasmid prior to the establishment of a new specificity.

Other evidence that RA is induced in response to the presence of unmodified target sequences on the chromosome of $hsdR^+M^+S^+$ bacteria, comes from the experiments of V.A.Doronina (Makovets *et al.*, 1999). It is found that a mutation in *hsdM*

(F269G) abolishes modification but not restriction activity of *Eco*KI. *hsdR*$^+$*M*$^-$ (F269G)*S*$^+$ bacteria are expected to die because of their r^+m^- phenotype. This was shown to be true only if the cells are *clp*$^-$. *clp*$^+$ cells survive due to constitutive ClpXP-dependent degradation of HsdR. These cells become depleted of HsdR and show the very low level of restriction that is a characteristic of RA. The only predicted difference between the *hsdR*$^+$*M*$^-$(F269G)*S*$^+$ and *hsdR*$^+$*M*$^+$*S*$^+$ bacteria is the absence of methylation in the first case, but it also results in the loss of restriction in a ClpXP-dependent manner.

The degradation of HsdR is inducible; under normal conditions the polypeptide is stable even in the presence of the ClpXP protease (Fig. 4.6). What is the signal that makes HsdR susceptible to proteolysis? This signal requires unmodified target recognition sites on the chromosome and a functional *Eco*KI complex; HsdR is stable in untreated cells (Fig. 4.5 & 4.6) as well as in bacteria where *Eco*KI is inactive either because of the absence of HsdM and HsdS or because of a point mutation in *hsdR* which inactivates the translocation and therefore the restriction activity of the endonuclease (Fig. 4.7). The requirement for unmodified targets and active nuclease suggests that DNA cleavage by *Eco*KI is necessary to induce RA. However, this hypothesis is contradicted by other data. First, *recA* bacteria, which would be expected to have problems with the repair of DSBs produced by *Eco*KI, are no more sensitive to 2-AP than wild-type cells, and show RA. This finding implies that DSBs do not occur during RA. Second, the experiments (Fig. 4.7b) with two systems (*Eco*KI and *Eco*AI) from different families suggest that the signal for the degradation of HsdR does not work *in trans*, in contrast to what would be expected if the signal were DSBs. RA for both *Eco*KI and *Eco*AI is regulated in a ClpXP-dependent manner. RA induced for the active *Eco*AI system does not lead to the degradation of HsdR from inactive *Eco*KI. Neither a diffusible molecule nor a DSB or other structure which might appear in the cell during RA is a signal for the degradation of HsdR. It would appear that the relevant signal depends on changes within the endonuclease complex itself.

The *hsdR* (A619V) mutation that abolishes the ClpXP-dependent degradation of HsdR does not affect the ability of the polypeptide to bind the methylase and form the $R_2M_2S_1$ complex. This $R_2M_2S_1$ complex recognises *Eco*KI target sequences and

binds them (Davies *et al.*, 1998). This mutation affects a DEAD-box motif that plays a role in ATP-dependent DNA translocation and the mutant enzyme fails to translocate (Davies *et al.*, 1999b). It is possible that *Eco*KI undergoes a conformational change when it translocates DNA and exposes an amino acid sequence that is recognised by ClpXP (Fig. 4.10). In agreement with this hypothesis, mutations in each of the other six DEAD-box motifs, all of which like *hsdR* (A619V) block DNA translocation (Davies *et al.*, 1999b) also abolish the ClpXP-dependent degradation of HsdR (V.A.Doronina, pers.comm.).

The relevance of the translocation step to the stability of HsdR is supported by the demonstration that mutations in *hsdR* that abolish the endonuclease, but not translocation activity of *Eco*KI (Davies *et al.*, 1999b), leave HsdR susceptible to proteolysis (V.A.Doronina, pers.comm.). Although DSBs cannot be produced by these mutant enzynes and there is no potential danger of chromosome breakage, the mutant is depleted of HsdR if the chromosome is unmodified. Modification activity is blocked by the *hsdM* (F269G) mutation. *Eco*KI of a double mutant (*hsdM* (F269G) + a mutation in the endonuclease motif) is expected to possess translocation activity but should neither methylate nor cut DNA. Lack of methylation results in unmethylated chromosomal target sequences, which are recognised by *Eco*KI. The DNA is translocated but cannot be cut because of the mutation in the endonuclease motif. The deficiency in endonuclease activity does not block the degradation of HsdR and this implies that the translocation activity is sufficient to promote proteolysis.

clpX has a more severe effect than *clpP* on the establishment of a new type I specificity (see Chapter 3) as if ClpX on its own helps cells deficient in the protease to survive. ClpX possesses a chaperone activity and can promote disassembly of multisubunit complexes (Levchenko *et al.*, 1997a). In the case of type I systems ClpX might remove HsdR from translocating complexes and prevent DNA cleavage. In the absence of ClpP, HsdR is likely to be released to the cytoplasm where it can bind M_2S_1 and form an active nuclease *de novo*. However, ClpX on its own is not as effective as the ClpXP protease in defending bacterial chromosomes from the breakage because, in the absence of ClpP, HsdR cannot be inactivated and most *clpP* cells still do not survive the establishment of *Eco*KI or *Eco*AI.

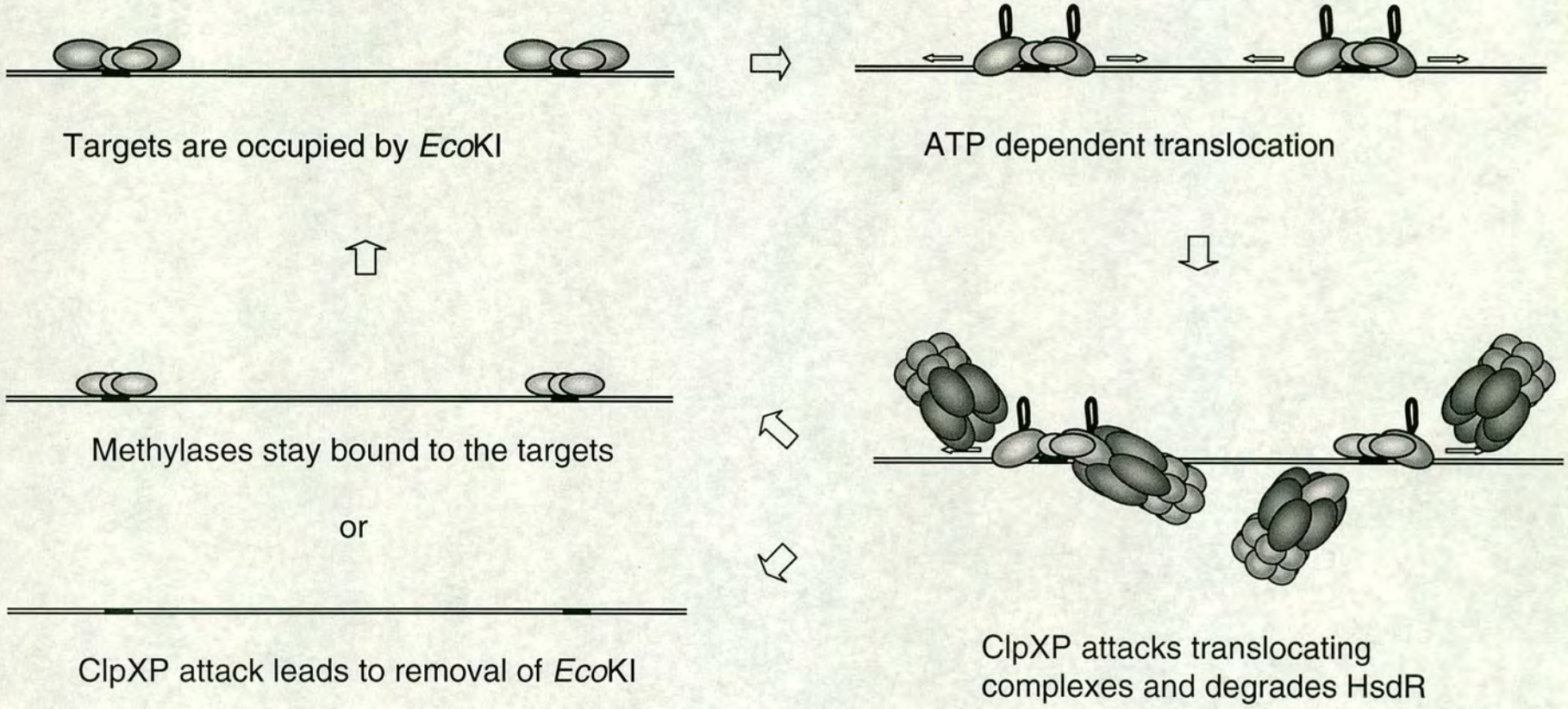


Fig. 4.10. Model representing the molecular mechanism of ClpXP-dependent degradation of HsdR. When unmodified target sequences are occupied by *Eco*KI and translocation takes place, ClpXP recognises HsdR, removes the R-subunits from translocating complexes and degrades them. The methyltransferase components of an original $R_2M_2S_1$ complex either remain bound to the target as a methylase or they are removed from DNA during the attack of ClpXP on HsdR. If the target stay unmethylated at the moment of the removal of HsdR it can be occupied by a nuclease again and stimulate degradation of other R-subunits.

CHAPTER 5. GENERAL DISCUSSION

Why do bacteria, some bacteriophages and bacterial plasmids encode restriction and modification systems? A well-established answer to this question is that R-M systems protect their hosts from incoming DNA and this can be of vital importance in some cases, e.g. phage infection. Similarly, R-M systems encoded by plasmids or temperate phages defend bacteria against invasion of foreign DNA but in doing so they also secure the reproduction of the replicons that encode them. Transfer of another plasmid from the same group of incompatibility could lead to elimination of the resident plasmid, whereas phage infection would kill the bacteria along with their resident replicons. It seems probable that the defensive role of R-M systems has made them widespread among bacteria and their transferable elements.

Kobayashi and co-workers have a different point of view on factors that influence the evolution of type II R-M systems. The finding that loss of genes specifying a type II R-M system results in cell death provoked an idea that these systems act as aggressors; once they have occupied a bacterium their genes have to be maintained and the restriction activity is mainly to kill those cells that lose their restriction and modification genes.

This idea is not readily applicable to type I systems since they have evolved a regulatory mechanism that secures the host bacteria from being killed by the restriction enzymes. The importance of this mechanism becomes clear when the regulation is blocked by a mutation in *clpX* or *clpP*. The ClpXP-dependent regulation permits the efficient acquisition of genes specifying type IA and IB R-M systems when they are transferred from one bacterium to another, and provides a basis for the distribution of type I systems via horizontal gene transfer.

Systems from the same family have a common ancestor and their differences in TRDs which define specificities are the result of divergent evolution. Change of specificity requires changes in the TRDs within HsdS. Probably, multiple mutations in a TRD-encoding DNA are required to evolve from one specificity to another. As a result, intermediate forms of the enzyme are likely to have no specificity: they have already lost the old specificity but have not acquired a new one. The last step in the process, the acquisition of a new specificity, is ClpXP dependent but very recent experiments show that the ClpXP protease is also important for the first step, that is

cell survival under some conditions of loss of specificity (S.Makovets & N.E.Murray, unpublished results). A particularly interesting case is a point mutation in *hsdS* of a type IA system which inactivates DNA-specific binding by the enzyme. Transfer of this mutation to *hsd*$^+$ *clpX* bacteria leads to a 25-fold drop in cell survival.

Some other point mutations in non-conserved regions of HsdS result in poor methylation but leave restriction functional and therefore they cannot be maintained in ClpXP-deficient cells (M.O'Neill & N.E.Murray, unpublished results). Mutations with similar phenotypes, also, could be intermediate steps in the evolution of new specificities. Regulation of restriction by ClpXP might play a role in evolution of type I specificities by permitting the systems to evolve through some forms of the enzymes that are lethal for bacteria in the absence of the protease.

Regulation of the endonuclease activity may eliminate not only the barriers to the mode of evolution of type I systems themselves but it may also prevent type I systems from limiting genetic change in those bacteria that possess them. In the absence of any control of endonuclease activity mutations that lead to the formation of new and therefore unmodified target sequences for type I enzymes in bacterial chromosomes would be excluded by their lethal effects. On the other hand, some mutations would result in the elimination of recognition sites from the chromosome. Under these conditions, the number of target sites recognised by a resident R-M system in the host chromosome would become greatly reduced during evolution. However, the number of *Eco*KI targets in the *E. coli* K-12 chromosome is very close to that calculated on the basis of random distribution of nucleotide sequences. This suggests that either the regulatory pathway appeared in the early stages of evolution of *E. coli* K-12 or that it originated in some other bacteria before the *hsd* genes were transferred to an ancestor of *E. coli* K-12.

Another role of the ClpXP-dependent regulation of type I enzymes might be relevant to bacterial survival under the conditions of the SOS response or methionine starvation. There are no direct data about the effect of *Eco*KI on the survival of ClpXP-deficient bacteria following UV treatment, but lethality of growth in the presence of 2-AP and in the case of the establishment of a new R-M system for *clp* mutants suggest that in situations when RA has to be induced the inability to do so is

lethal for bacteria (see 3.3.1 & 4.8). UV treatment also has been shown to induce RA and therefore it is very likely that the induction of the SOS response in *clp* bacteria will cause cell death. DNA repair via homologous recombination and UmuD′$_2$C-dependent replication will stimulate the appearance of unmodified targets in the chromosome (Fig. 4.9) which will be lethal in the absence of the protease.

The role of the regulation of type I endonucleases under the conditions of methionine starvation is rather speculative. Early experiments show that growth in the presence of ethionine, when bacteria are depleted of AdoMet, is lethal for cells encoding either type II or type III systems, but not type I (Lark & Arber, 1970). It was suggested that type I enzymes do not cleave host chromosomes because they require AdoMet for restriction, whereas neither type II nor type III require AdoMet and therefore will cleave the chromosome. An alternative explanation for these experiments can be suggested. A low concentration of AdoMet will result in under-methylation but it is likely to leave some level of the active nuclease bound to the cofactor. Under the conditions of under-methylation a few molecules of endonuclease loaded with AdoMet might be enough to damage the chromosome to a non-repairable state. However, this would be prevented by ClpXP-dependent degradation of HsdR and the cells would not die. A simple experiment might give an answer to this question; if ClpXP is involved in rescuing bacteria under the conditions of methionine starvation then *clpX* and *clpP* mutants will not survive growth in the presence of ethionine.

The discovery of the regulatory pathway for type I R-M systems has provided an explanation for some phenomena, such as the establishment of a new specificity and restriction alleviation, which have not been understood for years. At the same time this finding has complicated a well-established understanding of the biology of type I R-M systems. It was thought that only the methylation state of DNA is critical for its cleavage by restriction enzymes and this explained how the systems functioned; resident DNA is always methylated or hemimethylated and therefore it is not a substrate for restriction whereas incoming DNA, if it is unmethylated, is cleaved. However, resident DNA can also be unmethylated but it unlike unmethylated foreign DNA is not cleaved due to ClpXP. What is the difference between incoming and resident unmethylated DNA that allows cutting of the former and prevents cleavage of the latter? The restriction enzymes are "blind" because they cut both chromosomal

and phage unmodified DNA in the absence of ClpXP. Therefore, the factor that allows the discrimination is likely to affect the proteolysis of HsdR by ClpXP.

ClpXP is shown to regulate type IA and IB systems, two out of four known families. *Eco*R124I (type IC) and *Sty*SBLI (type ID) are also susceptible to 2-AP and respond by lowering restriction. 2-AP-induced RA of *Eco*R124I is not ClpXP-dependent, nor is it dependent on any other known cytoplasmic protease (S.Makovets & N.E.Murray, unpublished). Some preliminary data suggest that the stability of HsdR is not affected during RA. Apparently, the regulation of *Eco*R124I is based on some other mechanisms that might have similarity or even a common step with ClpXP-dependent regulation of type IA and IB systems. The finding that representatives of all four families can show RA implies that regulatory pathways preventing breakage of unmodified resident DNA by endonucleases might be a common characteristic of type I R-M systems.

REFERENCES

Adams G.M., Blumenthal R.M. (1995) Gene *pvuIIW*: a possible modulator of *Pvu*II endonuclease subunit association. *Gene* **157**: 193-199.

Akiyama Y., Yoshihisa T., Ito K. (1995) FtsH, a membrane-bound ATPase, forms a complex in the cytoplasmic membrane of *Escherichia coli*. *J. Biol. Chem.* **270**: 23485-23490.

Akiyama Y., Kihara A., Mori H., Ogura T., Ito K. (1998) Roles of the periplasmic domain of *Escherichia coli* FtsH (HflB) in protein interactions and activity modulation. *J. Biol. Chem.* **273**: 22326-22333.

Althorpe N.J., Chilley P.M., Thomas A.T., Brammar W.J., Wilkins B.M. (1999) Transient transcriptional activation of the IncI1 plasmid anti-restriction gene (*ardA*) and SOS inhibition gene (*psiB*) early in conjugating recipient bacteria. *Mol. Microbiol.* **31**: 133-142.

Appleyard R.K. (1954) Segregation of lambda lysogenicity during bacterial recombination in *Escherichia coli* K-12. *Genetics* **39**: 440-452.

Arber W., Dussoix D. (1962) Host specificity of DNA produced by *Escherichia coli*. I. Host controlled modification of bacteriophage lambda. *J. Mol. Biol.* **5**: 18-36.

Arber W., Linn S. (1969) DNA modification and restriction. *Annu. Rev. Biochem.* **38**: 467-500.

Arber W., Wauters-Willems D. (1970) Host specificity of DNA produced by *Escherichia coli*. XII. The two restriction and modification systems of strain 15T-. *Mol. Gen. Genet.* **108**: 203-217.

Arber W. (1974) DNA modification and restriction. *Prog. Nucleic Acid Res. Mol. Biol.* **14**: 1-37.

Bachi B., Reiser J., Pirrotta V. (1979) Methylation and cleavage sequences of the *Eco*P1 restriction-modification enzyme. *J. Mol. Biol.* **128**: 143-163.

Bandyopadhyay P.K., Studier F.W., Hamilton D.L., Yuan R. (1985) Inhibition of the type I restriction-modification enzymes *Eco*B and *Eco*K by the gene 0.3 protein of bacteriophage T7. *J. Mol. Biol.* **182**: 567-578.

Banuett F., Hoyt M.A., McFarlane L., Echols H., Herskowitz I. (1986) *hflB*, a new *Escherichia coli* locus regulating lysogeny and the level of bacteriophage lambda cII protein. *J. Mol. Biol.* **187**: 213-224.

Barcus V.A., Titheradge A.J., Murray N.E. (1995) The diversity of alleles at the hsd locus in natural populations of *Escherichia coli*. *Genetics* **140**: 1187-1197.

Beary T.P., Braymer H.D., Achberger E.C. (1997) Evidence of participation of McrB(S) in McrBC restriction in *Escherichia coli* K-12. *J. Bacteriol.* **179**: 7768-7775.

Becker G., Klauck E., Hengge-Aronis R. (1999) Regulation of RpoS proteolysis in Escherichia coli: the response regulator RssB is a recognition factor that interacts with the turnover element in RpoS. *Proc. Natl. Acad. Sci. USA* **96**: 6439-6444.

Belogurov A.A., Delver E.P., Rodzevich O.V. (1992) IncN plasmid pKM101 and IncI1 plasmid ColIb-P9 encode homologous antirestriction proteins in their leading regions. *J. Bacteriol.* **174**: 5079-5085.

Belogurov A.A., Delver E.P., Rodzevich O.V. (1993) Plasmid pKM101 encodes two nonhomologous antirestriction proteins (ArdA and ArdB) whose expression is controlled by homologous regulatory sequences. *J. Bacteriol.* **175**: 4843-4850.

Bertani G., Weigle J.J. (1953) Host controlled variation in bacterial viruses. *J. Bacteriol.* **65**: 113-121.

Bickle T.A., Kruger D.H. (1993) Biology of DNA restriction. *Microbiol. Rev.* **57**: 434-450.

Bitinaite J., Wah D.A., Aggarwal A.K., Schildkraut I. (1998) *Fok*I dimerization is required for DNA cleavage. *Proc. Natl. Acad. Sci. USA* **95**: 10570-10575.

Blumenthal R.M., Gregory S.A., Cooperider J.S. (1985) Cloning of a restriction-modification system from *Proteus vulgaris* and its use in analyzing a methylase-sensitive phenotype in *Escherichia coli. J. Bacteriol.* **164**: 501-509.

Bochtler M., Ditzel L., Groll M., Huber R. (1997) Crystal structure of heat shock locus V (HslV) from *Escherichia coli. Proc. Natl. Acad. Sci. USA* **94**: 6070-6074.

Bockrath R.C., Hanawalt P.C. (1980) Ultraviolet light induction of RecA protein in a *recB uvrB* mutant of *Escherichia coli. J. Bacteriol.* **143**: 1025-1028.

Bolivar F., Rodriguez R.L., Greene P.J., Betlach M.C., Heyneker H.L., Boyer H.W. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**: 95-113.

Bouche S., Klauck E., Fischer D., Lucassen M., Jung K., Hengge-Aronis R. (1998) Regulation of RssB-dependent proteolysis in *Escherichia coli*: a role for acetyl phosphate in a response regulator-controlled process. *Mol. Microbiol.* **27**: 787-795.

Boyer H. (1964) Genetic control of restriction and modification in *Escherichia coli. J. Bacteriol.* **88**: 1652-1660.

Boyer H.W., Roulland-Dussoix D. (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli. J. Mol. Biol.* **41**: 459-472.

Boyer H., Scibienski E., Slocum H., Roulland-Dussoix D. (1971) The *in vitro* restriction of the replicative form of W.T. and mutant fd phage DNA. *Virology* **46**: 703-10.

Brockes J.P., Brown P.R., Murray K. (1972) The deoxyribonucleic acid modification enzyme of bacteriophage P1. *Biochem J.* **127**: 1-10.

Brody H., Greener A., Hill C.W. (1985) Excision and reintegration of the *Escherichia coli* K-12 chromosomal element e14. *J. Bacteriol.* **161**: 1112-1117.

Brooks J.E., Benner J.S., Heiter D.F., Silber K.R., Sznyter L.A., Jager-Quinton T., Moran L.S., Slatko B.E., Wilson G.G., Nwankwo D.O. (1989) Cloning the *Bam*HI restriction modification system. *Nucleic Acids Res.* **17**: 979-997.

Brown N.L., Smith M. (1977) Cleavage specificity of the restriction endonuclease isolated from *Haemophilus gallinarum* (*Hga*I). *Proc. Natl. Acad. Sci. USA* **74**: 3213-3216.

Bullas L.R., Colson C. (1975) DNA restriction and modification systems in *Salmonella*. III. SP, a *Salmonella potsdam* system allelic to the SB system in *Salmonella typhimurium*. *Mol. Gen. Genet.* **139**: 177-188.

Bullas L.R., Colson C., Neufeld B. (1980) Deoxyribonucleic acid restriction and modification systems in *Salmonella*: chromosomally located systems of different serotypes. *J. Bacteriol.* **141**: 275-292.

Bullock W.O. *et al.* (1987) *BioTechniques* 5: 376-378.

Burckhardt S.E., Woodgate R., Scheuermann R.H., Echols H. (1988) UmuD mutagenesis protein of *Escherichia coli*: overproduction, purification, and cleavage by RecA. *Proc. Natl. Acad. Sci. USA* **85**: 1811-1815.

Chang A.C., Cohen S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. *J. Bacteriol.* **134**: 1141-1156.

Chater K.F., Wilde L.C. (1980) *Streptomyces albus* G mutants defective in the *Sal*GI restriction-modification system. *J. Gen. Microbiol.* **116**: 323-334.

Chen A., Powell L.M., Dryden D.T., Murray N.E., Brown T. (1995) Tyrosine 27 of the specificity polypeptide of *Eco*KI can be UV crosslinked to a bromodeoxyuridine-substituted DNA target sequence. *Nucleic Acids Res.* **23**: 1177-1183.

Chilley P.M., Wilkins B.M. (1995) Distribution of the *ardA* family of antirestriction genes on conjugative plasmids. *Microbiology* **141** : 2157-2164.

Chin D.T., Goff S.A., Webster T., Smith T., Goldberg A.L. (1988) Sequence of the *lon* gene in *Escherichia coli*: a heat-shock gene which encodes the ATP-dependent protease La. *J. Biol. Chem.* **263**: 11718-11728.

Chuang S.E., Burland V., Plunkett G., Daniels D.L., Blattner F.R. (1993) Sequence analysis of four new heat-shock genes constituting the *hslTS/ibpAB* and *hslVU* operons in *Escherichia coli*. *Gene* **134**: 1-6.

Clark A.J., Sharma V., Brenowitz S., Chu C.C., Sandler S., Satin L., Templin A., Berger I., Cohen A. (1993) Genetic and molecular analyses of the C-terminal region of the *recE* gene from the Rac prophage of *Escherichia coli* K-12 reveal the *recT* gene. *J. Bacteriol.* **175**: 7673-7682.

Colson C., Colson A.M., Van Pel A. (1970) Chromosomal location of host specificity in *Salmonella typhimurium*. *J. Gen. Microbiol.* **60**: 265-271.

Colson A.M., Colson C. (1972) Expression of the *Escherichia coli* K, B and phage P1 DNA host specificities in *Salmonella typhimurium*. *J. Gen. Microbiol.* **70**: 123-128.

Conrad M., Topal M.D. (1989) DNA and spermidine provide a switch mechanism to regulate the activity of restriction enzyme *Nae* I. *Proc. Natl. Acad. Sci. USA* **86**: 9707-9711.

Courcelle J., Carswell-Crumpton C., Hanawalt P.C. (1997) *recF* and *recR* are required for the resumption of replication at DNA replication forks in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **94**: 3714-3719.

Cowan G.M., Gann A.A., Murray N.E. (1989) Conservation of complex DNA recognition domains between families of restriction enzymes. *Cell* **56**: 103-109.

Cowlishaw J., Ginoza W. (1970) Induction of lambda prophage by nalidixic acid. *Virology* **41**: 244-255.

Dahlmann B., Kopp F., Kuehn L., Niedel B., Pfeifer G., Hegerl R., Baumeister W. (1989) The multicatalytic proteinase (prosome) is ubiquitous from eukaryotes to archaebacteria. *FEBS Lett.* **251**: 125-131.

Daniel A.S., Fuller-Pace F.V., Legge D.M., Murray N.E. (1988) Distribution and diversity of *hsd* genes in *Escherichia coli* and other enteric bacteria. *J. Bacteriol.* **170**: 1775-1782.

Dartois V., De Backer O., Colson C. (1993) Sequence of the *Salmonella typhimurium* StyLT1 restriction-modification genes: homologies with *Eco*P1 and *Eco*P15 type-III R-M systems and presence of helicase domains. *Gene* **127**: 105-110.

Davies G.P., Powell L.M., Webb J.L., Cooper L.P., Murray N.E. (1998) *Eco*KI with an amino acid substitution in any one of seven DEAD-box motifs has impaired ATPase and endonuclease activities. *Nucleic Acids Res.* **26**: 4828-4836.

Davies G.P., Martin I., Sturrock S.S., Cronshaw A., Murray N.E., Dryden D.T. (1999a) On the structure and operation of type I DNA restriction enzymes. *J. Mol. Biol.* **290**: 565-579.

Davies G.P., Kemp P., Molineux I.J., Murray N.E. (1999b) The DNA translocation and ATPase activities of restriction-deficient mutants of *Eco*KI. *J. Mol. Biol.* **292**: 787-796.

Day R.S. (1977) UV-induced alleviation of K-specific restriction of bacteriophage lambda. *J. Virol.* **21**: 1249-1251.

De Backer O., Colson C. (1991a) Two-step cloning and expression in *Escherichia coli* of the DNA restriction-modification system *Sty*LTI of *Salmonella typhimurium*. *J. Bacteriol.* **173**: 1321-1327.

De Backer O., Colson C. (1991b) Transfer of the genes for the *Sty*LTI restriction-modification system of *Salmonella typhimurium* to strains lacking modification ability results in death of the recipient cells and degradation of their DNA. *J. Bacteriol.* **173**: 1328-1330.

De Backer O., Colson C. (1991c) Identification of the recognition sequence for the M.StyLTI methyltransferase of *Salmonella typhimurium* LT7: an asymmetric site typical of type-III enzymes. *Gene* **97**: 103-107.

Defais M., Fauquet P., Radman M., Errera M. (1971) Ultraviolet reactivation and ultraviolet mutagenesis of lambda in different genetic systems. *Virology* **43**: 495-503.

Delver E.P., Kotova V.U., Zavilgelsky G.B, Belogurov A.A. (1991) Nucleotide sequence of the gene (*ard*) encoding the antirestriction protein of plasmid colIb-P9. *J. Bacteriol.* **173**: 5887-5892.

de Lorenzo V., Herrero M., Jakubzik U., Timmis K.N. (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172**: 6568-6572.

De Witt S.K., Adelberg E.A. (1962) The occurance of a genetic transposition in a strain of *Escherichia coli. Genetics.* **47**: 577.

Dharmalingam K., Goldberg E.B. (1980) Restriction *in vivo*. V. Introduction of SOS functions in *Escherichia coli* by restricted T4 phage DNA, and alleviation of restriction by SOS functions. *Mol. Gen. Genet.* **178**: 51-58.

Diaz R., Barnsley P., Pritchard R.H. (1979) Location and characterisation of a new replication origin in the *E. coli* K12 chromosome. *Mol. Gen. Genet.* **175**: 151-157.

Dila D., Sutherland E., Moran L., Slatko B., Raleigh E.A. (1990) Genetic and sequence organization of the *mcrBC* locus of *Escherichia coli* K-12. *J. Bacteriol.* **172**: 4888-4900.

Drlica K., Engle E.C., Manes S.H. (1980) DNA gyrase on the bacterial chromosome: possibility of two levels of action. *Proc. Natl. Acad. Sci. USA* **77**: 6879-6883.

Dryden D.T., Cooper L.P., Murray N.E. (1993) Purification and characterization of the methyltransferase from the type I restriction and modification system of *Escherichia coli* K12. *J. Biol. Chem.* **268**: 13228-13236.

Dryden D.T., Cooper L.P., Thorpe P.H., Byron O. (1997) The *in vitro* assembly of the *Eco*KI type I DNA restriction/modification enzyme and its *in vivo* implications. *Biochemistry* **36**: 1065-1076.

Dussoix D., Arber W. (1962) Host specificity of DNA produced by *Escherichia coli*. II. Control over acceptance of DNA from infecting phage lambda. *J. Mol. Biol.* **5**: 37-49.

Dybvig K., Sitaraman R., French C.T. (1998) A family of phase-variable restriction enzymes with differing specificities generated by high-frequency gene rearrangements. *Proc. Natl. Acad. Sci. USA* **95**: 13923-13928.

Echols H., Lu C., Burgers P.M. (1983) Mutator strains of *Escherichia coli, mutD* and *dnaQ*, with defective exonucleolytic editing by DNA polymerase III holoenzyme. *Proc. Natl. Acad. Sci. USA* **80**: 2189-2192.

Efimova E.P., Delver E.P., Belogurov A.A. (1988a) Alleviation of type I restriction in adenine methylase (*dam*) mutants of *Escherichia coli*. *Mol. Gen. Genet.* **214**: 313-316.

Efimova E.P., Delver E.P., Belogurov A.A. (1988b) 2-Aminopurine and 5-bromouracil induce alleviation of type I restriction in *Escherichia coli*: mismatches function as inducing signals? *Mol. Gen. Genet.* **214**: 317-320.

Engelberg-Kulka H., Reches M., Narasimhan S., Schoulaker-Schwarz R., Klemes Y., Aizenman E., Glaser G. (1998) *rexB* of bacteriophage lambda is an anti-cell death gene. *Proc. Natl. Acad. Sci. USA* **95**: 15481-15486.

Eskin B., Linn S. (1972) The deoxyribonucleic acid modification and restriction enzymes of *Escherichia coli* B. II. Purification, subunit structure, and catalytic properties of the restriction endonuclease. *J. Biol. Chem.* **247**: 6183-6191.

Fisher H., Glockbuster R. (1993) ATP hydrolysis is not stoichiometrically linked with proteolysis in the ATP-dependent protease La from *Escherichia coli*. *J. Biol. Chem.* **268**: 22502-22507.

Flanagan J.M., Wall J.S., Capel M.S., Schneider D.K., Shanklin J. (1995) Scanning transmission electron microscopy and small-angle scattering provide evidence that native *Escherichia coli* ClpP is a tetradecamer with an axial pore. *Biochemistry* **34**: 10910-10917.

Frank E.G., Ennis D.G., Gonzalez M., Levine A.S., Woodgate R. (1996) Regulation of SOS mutagenesis by proteolysis. *Proc. Natl. Acad. Sci. USA* **93**: 10291-10296.

Franklin N.C. (1985) Conservation of genome form but not sequence in the transcription antitermination determinants of bacteriophages lambda, phi 21 and P22. *J. Mol. Biol.* **181**: 75-84.

Freese E.B. (1968) The mutagenic effect of hydroxyaminopurine derivatives on phage T4. *Mutat. Res.* **5**: 299-301.

Fuller-Pace F.V., Bullas L.R., Delius H., Murray N.E. (1984) Genetic recombination can generate altered restriction specificity. *Proc. Natl. Acad. Sci. USA* **81**: 6095-6099.

Fuller-Pace F.V., Cowan G.M., Murray N.E. (1985) *Eco*A and *Eco*E: alternatives to the *Eco*K family of type I restriction and modification systems of *Escherichia coli*. *J. Mol. Biol.* **186**: 65-75.

Fuller-Pace F.V., Murray N.E. (1986) Two DNA recognition domains of the specificity polypeptides of a family of type I restriction enzymes. *Proc. Natl. Acad. Sci. USA* **83**: 9368-9372.

Gann A.A., Campbell A.J., Collins J.F., Coulson A.F., Murray N.E. (1987) Reassortment of DNA recognition domains and the evolution of new specificities. *Mol. Microbiol.* **1**: 13-22.

Gast F.U., Brinkmann T., Pieper U., Kruger T., Noyer-Weidner M., Pingoud A. (1997) The recognition of methylated DNA by the GTP-dependent restriction endonuclease McrBC resides in the N-terminal domain of McrB. *Biol. Chem.* **378**: 975-982.

Gellert M., Mizuuchi K., O'Dea M.H., Itoh T., Tomizawa J.I. (1977) Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. *Proc. Natl. Acad. Sci. USA* **74**: 4772-4776.

Geuskens V., Mhammedi-Alaoui A., Desmet L., Toussaint A. (1992) Virulence in bacteriophage Mu: a case of trans-dominant proteolysis by the *Escherichia coli* Clp serine protease. *EMBO J.* **11**: 5121-5127.

Glover S.W., Colson C. (1965) The breakdown of the restriction mechanism in zygotes of *Escherichia coli*. *Genet. Res.* **6**: 153-155.

Glover S.W., Colson C. (1969) Genetics of host-controlled restriction and modification in *Escherichia coli. Genet. Res.* **13**: 227-240.

Glover S.W. (1970) Functional analysis of host-specificity mutants in *Escherichia coli. Genet. Res.* **15**: 237-250.

Goff S.A., Casson L.P., Goldberg A.L. (1984) Heat shock regulatory gene *htpR* influences rates of protein degradation and expression of the *lon* gene *in Escherichia coli. Proc. Natl. Acad. Sci. USA* **81**: 6647-6651.

Goldberg A., Morschell R.P., Chung C.H., Maurizi M.R. (1994) ATP-dependent protease La (lon) from *Escherichia coli. Meth. Enzymol.* **244**: 350-375.

Gonciarz-Swiatek M., Wawrzynow A., Um S.J., Learn B.A., McMacken R., Kelley W.L., Georgopoulos C., Sliekers O., Zylicz M. (1999) Recognition, targeting, and hydrolysis of the lambda O replication protein by the ClpP/ClpX protease. *J. Biol. Chem.* **274**: 13999-14005.

Gonzalez M., Frank E.G., Levine A.S., Woodgate R. (1998) Lon-mediated proteolysis of the *Escherichia coli* UmuD mutagenesis protein: *in vitro* degradation and identification of residues required for proteolysis. *Genes Dev.***12**: 3889-3899.

Gorbalenya A.E., Koonin E.V., Donchenko A.P., Blinov V.M. (1988) A conserved NTP-motif in putative helicases. *Nature* **333**: 22.

Gorbalenya A.E., Koonin E.V. (1991) Endonuclease (R) subunits of type-I and type-III restriction-modification enzymes contain a helicase-like domain. *FEBS Lett.* **291**: 277-281.

Gottesman M.M., Gottesman M.E., Gottesman S., Gellert M. (1974) Characterization of bacteriophage lambda reverse as an *Escherichia coli* phage carrying a unique set of host-derived recombination functions. *J. Mol. Biol.* **88**: 471-487.

Gottesman S., Gottesman M., Shaw J.E., Pearson M.L. (1981) Protein degradation in *E. coli*: the *lon* mutation and bacteriophage lambda N and cll protein stability. *Cell* **24**: 225-233.

Gottesman S. (1990) Minimizing proteolysis in *Escherichia coli*: genetic solutions. *Methods Enzymol.* **185**: 119-129.

Gottesman S., Clark W.P., Maurizi M.R. (1990) The ATP-dependent Clp protease of *Escherichia coli*. Sequence of *clpA* and identification of a Clp-specific substrate. *J. Biol. Chem.* **265**: 7886-7893.

Gottesman S., Clark W.P., de Crecy-Lagard V., Maurizi M.R. (1993) ClpX, an alternative subunit for the ATP-dependent Clp protease of *Escherichia coli*. Sequence and *in vivo* activities. *J. Biol. Chem.* **268**: 22618-22626.

Gottesman S. (1996) Proteases and their targets *in Escherichia coli. Annu. Rev. Genet.* **30**: 465-506.

Gottesman S., Roche E., Zhou Y., Sauer R.T. (1998) The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev.* **12**: 1338-1347.

Gottesman S. (1999) Regulation by proteolysis: developmental switches. *Curr. Opin. Microbiol.* **2**: 142-147.

Gottschling H., Freese E. (1961) Incorporation of 2-aminopurine into the deoxyribonucleic acid of bacteria and bacteriophages. *Z. Naturforsch.* **16b**: 515-519.

Gough J.A., Murray N.E. (1983) Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J. Mol. Biol.* **166**: 1-19.

Granger L.L., O'Hara E.B., Wang R.F., Meffen F.V., Armstrong K., Yancey S.D., Babitzke P., Kushner S.R. (1998) The *Escherichia coli mrsC* gene is required for cell growth and mRNA decay. *J. Bacteriol.* **180**: 1920-1928.

Grimaud R., Kessel M., Beuron F., Steven A.C., Maurizi M.R. (1998) Enzymatic and structural similarities between the *Escherichia coli* ATP-dependent proteases, ClpXP and ClpAP. *J. Biol. Chem.* **273**: 12476-12481.

Gubler M., Braguglia D., Meyer J., Piekarowicz A., Bickle T.A. (1992) Recombination of constant and variable modules alters DNA sequence recognition by type IC restriction-modification enzymes. *EMBO J.* **11**: 233-240.

Gudas L.J., Pardee A.B. (1976) DNA synthesis inhibition and the induction of protein X in *Escherichia coli. J. Mol. Biol.* **101**: 459-477.

Gunthert U., Freund M., Trautner T.A. (1981) Restriction and modification in *Bacillus subtilis*: two DNA methyltransferases with *Bsu*RI specificity. I. Purification and physical properties. *J. Biol. Chem.* **256**: 9340-9345.

Haberman A., Heywood J., Meselson M. (1972) DNA modification methylase activity of *Escherichia coli* restriction endonucleases K and P. *Proc. Natl. Acad. Sci. USA* **69**: 3138-3141.

Haberman A. (1974) The bacteriophage P1 restriction endonuclease. *J. Mol. Biol.* **89**: 545-563.

Hadi S.M., Bachi B., Shepherd J.C., Yuan R., Ineichen K., Bickle T.A. (1979) DNA recognition and cleavage by the *Eco*P15 restriction endonuclease. *J. Mol. Biol.* **134**: 655-666.

Hegna I.K., Karlstrom E.S., Lopez R., Kristensen T., Kolsto A.B. (1992) A type-III DNA restriction and modification system in *Bacillus cereus*? *Gene* **114**: 149-150.

Heitman J., Model P. (1987) Site-specific methylases induce the SOS DNA repair response in *Escherichia coli. J. Bacteriol.* **169**: 3243-3250.

Heitman J., Treisman J., Davis N.G., Russel M. (1989) Cassettes of the f1 intergenic region. *Nucleic Acids Res.* **17**: 4413.

Herman C., Thevenet D., D'Ari R., Bouloc P. (1995) Degradation of sigma 32, the heat shock regulator in *Escherichia coli*, is governed by HflB. *Proc. Natl. Acad. Sci. USA* **92**: 3516-3520.

Herman C., Thevenet D., Bouloc P., Walker G.C., D'Ari R. (1998) Degradation of carboxy-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease HflB (FtsH). *Genes Dev.* **12**: 1348-1355.

Highlander S.K., Garza O. (1996) The restriction-modification system *of Pasteurella haemolytica* is a member of a new family of type I enzymes. *Gene* **178**: 89-96.

Hilliard J.J., Maurizi M.R., Simon L.D. (1998a) Isolation and characterization of the phage T4 PinA protein, an inhibitor of the ATP-dependent Lon protease of *Escherichia coli. J. Biol. Chem.* **273**: 518-523.

Hilliard J.J., Simon L.D., Van Melderen L., Maurizi M.R. (1998b) PinA inhibits ATP hydrolysis and energy-dependent protein degradation by Lon protease. *J. Biol. Chem.* **273**: 524-527.

Hiom K., Sedgwick S.G. (1991) Cloning and structural characterization of the *mcrA* locus of *Escherichia coli. J. Bacteriol.* **173**: 7368-7373.

Hiom K., Thomas S.M., Sedgwick S.G. (1991) Different mechanisms for SOS induced alleviation of DNA restriction in *Escherichia coli. Biochimie* **73**: 399-405.

Hiom K.J., Sedgwick S.G. (1992) Alleviation of *Eco*K DNA restriction in *Escherichia coli* and involvement of *umuDC* activity. *Mol. Gen. Genet.* **231**: 265-275.

Hirsch J.A., Wah D.A., Dorner L.F., Schildkraut I., Aggarwal A.K. (1997) Crystallization and preliminary X-ray analysis of restriction endonuclease *Fok*I bound to DNA. *FEBS Lett.* **403**: 136-138.

Horiuchi K., Zinder N.D. (1972) Cleavage of bacteriophage fl DNA by the restriction enzyme of *Escherichia coli* B. *Proc. Natl. Acad. Sci. USA* **69**: 3220-3224.

Hoskins J.R., Pak M., Maurizi M.R., Wickner S. (1998) The role of the ClpA chaperone in proteolysis by ClpAP. *Proc. Natl. Acad. Sci. USA* **95**: 12135-12140.

Howard K.A., Card C., Benner J.S., Callahan H.L., Maunus R., Silber K., Wilson G., Brooks J.E. (1986) Cloning the *Dde*I restriction-modification system using a two-step method. *Nucleic Acids Res* **14**: 7939-7951.

Humbelin M., Suri B., Rao D.N., Hornby D.P., Eberle H., Pripfl T., Kenel S., Bickle T.A. (1988) Type III DNA restriction and modification systems *Eco*P1 and *Eco*P15. Nucleotide sequence of the *Eco*P1 operon, the *Eco*P15 *mod* gene and some *Eco*P1 *mod* mutants. *J. Mol. Biol.* **200**: 23-29.

Iida S., Meyer J., Bachi B., Stalhammar-Carlemalm M., Schrickel S., Bickle T.A., Arber W. (1983) DNA restriction--modification genes of phage P1 and plasmid p15B. Structure and *in vitro* transcription. *J. Mol. Biol.* **165**: 1-18.

Iida S., Streiff M.B., Bickle T.A., Arber W. (1987) Two DNA antirestriction systems of bacteriophage P1, *darA*, and *darB*: characterization of *darA*- phages. *Virology* **157**: 156-166.

Ives C.L, Nathan P.D., Brooks J.E. (1992) Regulation of the *Bam*HI restriction-modification system by a small intergenic open reading frame, *bamHIC*, in both *Escherichia coli* and *Bacillus subtilis*. *J. Bacteriol.* **174**: 7194-7201.

Ives C.L., Sohail A., Brooks J.E. (1995) The regulatory C proteins from different restriction-modification systems can cross-complement. *J. Bacteriol.* **177**: 6313-6315.

Jacob & Wallman (1961) *Sexuality and the Genetics of Bacteria.* Academic Press, Inc., New York.

Janscak P., Dryden D.T., Firman K. (1998) Analysis of the subunit assembly of the type IC restriction-modification enzyme *Eco*R124I. *Nucleic Acids Res.* **26**: 4439-4445.

Janscak P., MacWilliams M.P., Sandmeier U., Nagaraja V., Bickle T.A. (1999a) DNA translocation blockage, a general mechanism of cleavage site selection by type I restriction enzymes. *EMBO J.* **18**: 2638-2647.

Janscak P., Sandmeier U., Bickle T.A. (1999b) Single amino acid substitutions in the HsdR subunit of the type IB restriction enzyme *Eco*AI uncouple the DNA translocation and DNA cleavage activities of the enzyme. *Nucleic Acids Res.* **27**: 2638-2643.

Janulaitis A., Petrusyte M., Maneliene Z., Klimasauskas S., Butkus V. (1992) Purification and properties of the *Eco*57I restriction endonuclease and methylase--prototypes of a new class (type IV). *Nucleic Acids Res.* **20**: 6043-6049.

Kaczorowski T., Skowron P., Podhajska A.J. (1989) Purification and characterization of the *Fok*I restriction endonuclease. *Gene* **80**: 209-216.

Kaiser K., Murray N.E. (1979) Physical characterisation of the "Rac prophage" in *E. coli* K12. *Mol. Gen. Genet.* **175**: 159-174.

Kan N.C., Lautenberger J.A., Edgell M.H., Hutchison C.A. (1979) The nucleotide sequence recognized by the *Escherichia coli* K12 restriction and modification enzymes. *J. Mol. Biol.* **130**: 191-209.

Kanemori M., Nishihara K., Yanagi H., Yura T. (1997) Synergistic roles of HslVU and other ATP-dependent proteases in controlling *in vivo* turnover of sigma32 and abnormal proteins in *Escherichia coli. J. Bacteriol.* **179**: 7219-7225.

Karu A.E., Belk E.D. (1982) Induction of *E. coli* recA protein via recBC and alternate pathways: quantitation by enzyme-linked immunosorbent assay (ELISA). *Mol. Gen. Genet.* **185**: 275-282.

Karyagina A., Shilov I., Tashlitskii V., Khodoun M., Vasil'ev S., Lau P.C.K., Nikolskaya I. (1997) Specific binding of Sso II DNA methyltransferase to its promoter region provides the regulation of *sso* II restriction-modification gene expression. *Nucleic Acids Res* **25**: 2114-2120.

Katayama Y., Gottesman S., Pumphrey J., Rudikoff S., Clark W.P., Maurizi M.R. (1988) The two-component, ATP-dependent Clp protease of *Escherichia coli.* Purification, cloning, and mutational analysis of the ATP-binding component. *J. Biol. Chem.* **263**: 15226-15236.

Kauc L., Piekarowicz A. (1978) Purification and properties of a new restriction endonuclease from *Haemophilus influenzae* Rf. *Eur. J. Biochem.* **92**: 417-426.

Keiler K.C., Waller P.R., Sauer R.T. (1996) Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science* **271**: 990-993.

Kelleher J.E., Raleigh E.A. (1991) A novel activity in *Escherichia coli* K-12 that directs restriction of DNA modified at CG dinucleotides. *J. Bacteriol.* **173**: 5220-5223.

Kelleher J.E., Daniel A.S., Murray N.E. (1991) Mutations that confer *de novo* activity upon a maintenance methyltransferase. *J. Mol. Biol.* **221**: 431-440.

Kelleher J.E., Raleigh E.A. (1994) Response to UV damage by four *Escherichia coli* K-12 restriction systems. *J. Bacteriol.* **176**: 5888-5896.

Kessel M., Maurizi M.R., Kim B., Kocsis E., Trus B.L. Singh S.K., Steven A.C. (1995) Homology in structural organization between *E. coli* ClpAP protease and the eukaryotic 26 S proteasome. *J. Mol. Biol.* **250**: 587-594.

Kessel M., Wu W., Gottesman S., Kocsis E., Steven A.C., Maurizi M.R. (1996) Six-fold rotational symmetry of ClpQ, the *E. coli* homolog of the 20S proteasome, and its

ATP-dependent activator, ClpY. *FEBS Lett.* **398**: 274-278.

Khattar M.M. (1997) Overexpression of the *hslVU* operon suppresses SOS-mediated inhibition of cell division in *Escherichia coli. FEBS Lett.* **414**: 402-404.

Kihara A., Akiyama Y., Ito K. (1995) FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. *Proc. Natl. Acad. Sci. USA* **92**: 4532-4536.

Kihara A., Akiyama Y., Ito K. (1996) A protease complex in the *Escherichia coli* plasma membrane: HflKC (HflA) forms a complex with FtsH (HflB), regulating its proteolytic activity against SecY. *EMBO J.* **15**: 6122-6131.

Kihara A., Akiyama Y., Ito K. (1998) Different pathways for protein degradation by the FtsH/HflKC membrane-embedded protease complex: an implication from the interference by a mutant form of a new substrate protein, YccA. *J. Mol. Biol.* **279**: 175-188.

Kim K.I., Woo K.M., Seong I.S., Lee Z.W., Baek S.H., Chung C.H. (1998) Mutational analysis of the two ATP-binding sites in ClpB, a heat shock protein with protein-activated ATPase activity in *Escherichia coli. Biochem. J.* **333**: 671-676.

Kim Y.C., Grable J.C., Love R., Greene P.J., Rosenberg J.M. (1990) Refinement of *Eco* RI endonuclease crystal structure: a revised protein chain tracing. *Science* **249**: 1307-1309.

King G., Murray N.E. (1995) Restriction alleviation and modification enhancement by the Rac prophage of *Escherichia coli* K-12. *Mol. Microbiol.* **16**: 769-777.

Kitagawa M., Wada C., Yoshioka S., Yura T. (1991) Expression of ClpB, an analog of the ATP-dependent protease regulatory subunit in *Escherichia coli*, is controlled by a heat shock sigma factor (sigma 32). *J. Bacteriol.* **173**: 4247-4253.

Kleid D., Humayun Z., Jeffrey A., Ptashne M. (1976) Novel properties of a restriction endonuclease isolated *from Haemophilus parahaemolyticus. Proc. Natl. Acad. Sci. USA* **73**: 293-297.

Koffel-Schwartz N., Coin F., Veaute X., Fuchs R.P. (1996) Cellular strategies for accommodating replication-hindering adducts in DNA: control by the SOS response in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **93**: 7805-7810.

Kohara Y., Akiyama K., Isono K. (1987) The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50**: 495-508.

Koncz C., Kiss A., Venetianer P. (1978) Biochemical characterization of the restriction-modification system of *Bacillus sphaericus. Eur. J. Biochem.* **89**: 523-529.

Konieczny I., Helinski D.R. (1997) The replication initiation protein of the broad-host-range plasmid RK2 is activated by the ClpX chaperone. *Proc. Natl. Acad. Sci. USA* **94**: 14378-14382.

Kretz P.L., Kohler S.W., Short J.M. (1991) Identification and characterization of a gene responsible for inhibiting propagation of methylated DNA sequences in *mcrA mcrB1 Escherichia coli* strains. *J. Bacteriol.* **173**: 4707-4716.

Kroger M., Hobom G. (1984) The nucleotide recognized by the *Escherichia coli* A restriction and modification enzyme. *Nucleic Acids Res.* **12**: 887-899.

Kroh H.E., Simon L.D. (1990) The ClpP component of Clp protease is the sigma 32-dependent heat shock protein F21.5. *J. Bacteriol.* **172**: 6026-6034.

Kruger D.H., Bickle T.A. (1983) Bacteriophage survival: multiple mechanisms for avoiding the deoxyribonucleic acid restriction systems of their hosts. *Microbiol. Rev.* **47**: 345-360.

Kruger D.H., Schroeder C., Reuter M., Bogdarina I.G., Buryanov Y.I., Bickle T.A. (1985) DNA methylation of bacterial viruses T3 and T7 by different DNA methylases in *Escherichia coli* K12 cells. *Eur. J. Biochem.* **150**: 323-330.

Kruger D.H., Barcak G.J., Reuter M., Smith H.O. (1988) *Eco*RII can be activated to cleave refractory DNA recognition sites. *Nucleic Acids Res.* **16**: 3997-4008.

Kruger T., Grund C., Wild C., Noyer-Weidner M. (1992) Characterization of the *mcrBC* region of *Escherichia coli* K-12 wild-type and mutant strains. *Gene* **114**: 1-12.

Kruger T., Wild C., Noyer-Weidner M. (1995) McrB: a prokaryotic protein specifically recognizing DNA containing modified cytosine residues. *EMBO J.* **14**: 2661-2669.

Kruklitis R., Welty D.J., Nakai H. (1996) ClpX protein of *Escherichia coli* activates bacteriophage Mu transposase in the strand transfer complex for initiation of Mu DNA synthesis. *EMBO J.* **15**: 935-944.

Kuhnlein U., Arber W. (1972) Host specificity of DNA produced by *Escherichia coli*. XV. The role of nucleotide methylation in *in vitro* B-specific modification. *J. Mol. Biol.* **63**: 9-19.

Kulakauskas S., Lubys A., Ehrlich S.D. (1995) DNA restriction-modification systems mediate plasmid maintenance. *J. Bacteriol.* **177**: 3451-3454.

Kulik E.M., Bickle T.A. (1996) Regulation of the activity of the type IC *Eco*R124I restriction enzyme. *J. Mol. Biol.* **264**: 891-906.

Kunz A., Mackeldanz P., Mucke M., Meisel A., Reuter M., Schroeder C., Kruger D.H. (1998) Mutual activation of two restriction endonucleases: interaction of *Eco*P1 and *Eco*P15. *Biol. Chem.* **379**: 617-620.

Kushner S.R., Nagaishi H., Templin A., Clark A.J. (1971) Genetic recombination in *Escherichia coli*: the role of exonuclease I. *Proc. Natl. Acad. Sci. USA* **68**: 824-827.

Laachouch J.E., Desmet L., Geuskens V., Grimaud R., Toussaint A. (1996) Bacteriophage Mu repressor as a target for the *Escherichia coli* ATP-dependent Clp Protease. *EMBO J.* **15**: 437-444.

Lacks S., Greenberg B. (1977) Complementary specificity of restriction endonucleases of *Diplococcus pneumoniae* with respect to DNA methylation. *J. Mol. Biol.* **114**: 153-168.

Lacks S.A., Mannarelli B.M., Springhorn S.S., Greenberg B. (1986) Genetic basis of the complementary *Dpn*I and *Dpn*II restriction systems of *S. pneumoniae*: an intercellular cassette mechanism. *Cell* **46**: 993-1000.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lange R., Hengge-Aronis R. (1994) The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes Dev.* **8**: 1600-1612.

Lark C., Arber W. (1970) Host specificity of DNA produced by *Escherichia coli*. 13. Breakdown of cellular DNA upon growth in ethionine of strains with r plus-15, r plus-P1 or r plus-N3 restriction phenotypes. *J. Mol. Biol.* **52**: 337-348.

Laskowska E., Kuczynska-Wisnik D., Skorko-Glonek J., Taylor A. (1996) Degradation by proteases Lon, Clp and HtrA, of *Escherichia coli* proteins aggregated in vivo by heat shock; HtrA protease action *in vivo* and *in vitro*. *Mol. Microbiol.* **22**: 555-571.

Lauster R. (1989) Evolution of type II DNA methyltransferases. A gene duplication model. *J. Mol. Biol.* **206**: 313-321.

Lauster R., Trautner T.A., Noyer-Weidner M. (1989) Cytosine-specific type II DNA methyltransferases. A conserved enzyme core with variable target-recognizing domains. *J. Mol. Biol.* **206**: 305-312.

Lautenberger J.A., Linn S. (1972) The deoxyribonucleic acid modification and restriction enzymes of *Escherichia coli* B. I. Purification, subunit structure, and catalytic properties of the modification methylase. *J. Biol. Chem.* **247**: 6176-6182.

Lautenberger J.A., Kan N.C., Lackey D., Linn S., Edgell M.H., Hutchison C.A. (1978) Recognition site of *Escherichia coli* B restriction enzyme on phi XsB1 and

simian virus 40 DNAs: an interrupted sequence. *Proc. Natl. Acad. Sci. USA* **75**: 2271-2275.

Lawrence C.W., Borden A., Banerjee S.K., LeClerc J.E. (1990) Mutation frequency and spectrum resulting from a single abasic site in a single-stranded vector. *Nucleic Acids Res.* **18**: 2153-2157.

Lee N.S., Rutebuka O., Arakawa T., Bickle T.A., Ryu J. (1997) *Kpn*AI, a new type I restriction-modification system in *Klebsiella pneumoniae. J. Mol. Biol.* **271**: 342-348.

Leffers G.G., Gottesman S. (1998) Lambda Xis degradation *in vivo* by Lon and FtsH. *J. Bacteriol.* **180**: 1573-1577.

Lehnherr H., Yarmolinsky M.B. (1995) Addiction protein Phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of *Escherichia coli. Proc. Natl. Acad. Sci. USA* **92**: 3274-3277.

Levchenko I., Luo L., Baker T.A. (1995) Disassembly of the Mu transposase tetramer by the ClpX chaperone. *Genes Dev.* **9**: 2399-2408.

Levchenko I., Yamauchi M., Baker T.A. (1997a) ClpX and MuB interact with overlapping regions of Mu transposase: implications for control of the transposition pathway. *Genes Dev.* **11**: 1561-1572.

Levchenko I., Smith C.K., Walsh N.P., Sauer R.T., Baker T.A. (1997) PDZ-like domains mediate binding specificity in the Clp/Hsp100 family of chaperones and protease regulatory subunits. *Cell* **91**: 939-947.

Linn S., Arber W. (1968) Host specificity of DNA produced by Escherichia coli, X. *In vitro* restriction of phage fd replicative form. *Proc. Natl. Acad. Sci. USA* **59**: 1300-1306.

Loenen W.A., Murray N.E. (1986) Modification enhancement by the restriction alleviation protein (Ral) of bacteriophage lambda. *J. Mol. Biol.* **190**: 11-22.

Loenen W.A., Daniel A.S., Braymer H.D., Murray N.E. (1987) Organization and sequence of the *hsd* genes of *Escherichia coli* K-12. *J. Mol. Biol.* **198**: 159-170.

Looney M.C., Moran L.S., Jack W.E., Feehery G.R., Benner J.S., Slatko B.E., Wilson G.G. (1989) Nucleotide sequence of the *Fok*I restriction-modification system: separate strand-specificity domains in the methyltransferase. *Gene* **80**: 193-208.

Low B. (1968) Formation of merodiploids in matings with a class of Rec- recipient strains of *Escherichia coli* K12. *Proc. Natl. Acad. Sci. USA* **60**: 160-167.

Low K.B. (1972) *Escherichia coli* K-12 F-prime factors, old and new. Bacteriol. Rev. 36: 587-607.

Low B. (1973) Restoration by the *rac* locus of recombinant forming ability in *recB* - and *recC* - merozygotes of *Escherichia coli* K-12. *Mol. Gen. Genet.* **122**: 119-130.

Lubys A., Lubiene J., Kulakauskas S., Stankevicius K., Timinskas A., Janulaitis A. (1996) Cloning and analysis of the genes encoding the type IIS restriction-modification system *Hph*I from Haemophilus parahaemolyticus. *Nucleic Acids Res.* **24**: 2760-1766.

Luria S.E., Human M.L. (1952) A nonhereditary, host-induced variation of bacterial viruses. *J. Bacteriol.* **64**: 557-569.

MacNeil D.J. (1988) Characterization of a unique methyl-specific restriction system in *Streptomyces avermitilis. J. Bacteriol.* **170**: 5607-5612.

Makovets S., Doronina V.A., Murray N.E. (1999) Regulation of endonuclease activity by proteolysis prevents breakage of unmodified bacterial chromosomes by type I restriction enzymes. *Proc. Natl. Acad. Sci. USA* **96**: 9757-9762.

Masters M., Paterson T., Popplewell A.G., Owen-Hughes T., Pringle J.H., Begg K.J. (1989) The effect of DnaA protein levels and the rate of initiation at *oriC* on transcription originating in the *ftsQ* and *ftsA* genes: *in vivo* experiments. *Mol. Gen. Genet.* **216**: 475-483.

Maurizi M.R., Clark W.P., Katayama Y., Rudikoff S., Pumphrey J., Bowers B., Gottesman S. (1990a) Sequence and structure of Clp P, the proteolytic component of the ATP-dependent Clp protease of *Escherichia coli. J. Biol. Chem.* **265**: 12536-12545.

Maurizi M.R., Clark W.P., Kim S.H., Gottesman S. (1990b) Clp P represents a unique family of serine proteases. *J. Biol. Chem.* **265**: 12546-12552.

McDonald J.P., Frank E.G., Levine A.S., Woodgate R. (1998) Intermolecular cleavage by UmuD-like mutagenesis proteins. *Proc. Natl. Acad. Sci. USA* **95**: 1478-1483.

McPartland A., Green L., Echols H. (1980) Control of *recA* gene RNA in *E. coli*: regulatory and signal genes. *Cell* **20**: 731-737.

Meisel A., Kruger D.H., Bickle T.A. (1991) M.*Eco*P15 methylates the second adenine in its recognition sequence. *Nucleic Acids Res.* **19**: 3997.

Meisel A., Bickle T.A., Kruger D.H., Schroeder C. (1992) Type III restriction enzymes need two inversely oriented recognition sites for DNA cleavage. *Nature* **355**: 467-469.

Meisel A., Mackeldanz P., Bickle T.A., Kruger D.H., Schroeder C. (1995) Type III restriction endonucleases translocate DNA in a reaction driven by recognition site-specific ATP hydrolysis. *EMBO J.* **14**: 2958-2966.

Meselson M., Yuan R. (1968) DNA restriction enzyme from *E. coli. Nature* **217**: 1110-1114.

Meselson M., Yuan R., Heywood J. (1972) Restriction and modification of DNA. *Annu. Rev. Biochem.* **41**: 447-466.

Mhammedi-Alaoui A., Pato M., Gama M.J., Toussaint A. (1994) A new component of bacteriophage Mu replicative transposition machinery: the *Escherichia coli* ClpX protein. *Mol. Microbiol.* **11**: 1109-1116.

Michel B., Ehrlich S.D., Uzest M. (1997) DNA double-strand breaks caused by replication arrest. *EMBO J.* **16**: 430-438.

Miller J.H. (1972) *Experiments in molecular genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miller J.H., Low K.B. (1984) Specificity of mutagenesis resulting from the induction of the SOS system in the absence of mutagenic treatment. *Cell* **37**: 675-682.

Missiakas D., Schwager F., Betton J.M., Georgopoulos C., Raina S. (1996) Identification and characterization of HsIV HsIU (ClpQ ClpY) proteins involved in overall proteolysis of misfolded proteins in *Escherichia coli. EMBO J.* **15**: 6899-6909.

Miura A., Tomizawa J.I. (1968) Studies on radiation-sensitive mutants of *E. coli.* 3. Participation of the *rec* system in induction of mutation by ultraviolet irradiation. *Mol. Gen. Genet.* **103**: 1-10.

Mizusawa S., Gottesman S. (1983) Protein degradation in *Escherichia coli*: the *lon* gene controls the stability of *sulA* protein. *Proc. Natl. Acad. Sci. USA* **80**: 358-362.

Mizuuchi K. (1992) Polynucleotidyl transfer reactions in transpositional DNA recombination. *J. Biol. Chem.* **267**: 21273-21276.

Motohashi K., Watanabe Y., Yohda M., Yoshida M. (1999) Heat-inactivated proteins are rescued by the DnaK.J-GrpE set and ClpB chaperones. *Proc. Natl. Acad. Sci. USA* **96**: 7184-7189.

Muffler A., Fischer D., Altuvia S., Storz G., Hengge-Aronis R. (1996) The response regulator RssB controls stability of the sigma (S) subunit of RNA polymerase in *Escherichia coli. EMBO J.* **15**: 1333-1339.

Murray N.E., Brammar W.J., Murray K. (1977) Lambdoid phages that simplify the recovery of *in vitro* recombinants. *Mol. Gen. Genet.* **150**: 53-61.

Murray N.E., Gough J.A., Suri B., Bickle T.A. (1982) Structural homologies among type I restriction-modification systems. *EMBO J.* **1**: 535-539.

Murray N.E. (1983) Phage lambda and molecular cloning. In *Lambda II.* Hendrix R.W., Roberts J.W., Stahl F.W., & Weisberg R.A. (eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Murray N.E., Daniel A.S., Cowan G.M., Sharp P.M. (1993) Conservation of motifs within the unusually variable polypeptide sequences of type I restriction and modification enzymes. *Mol. Microbiol.* **9**: 133-143.

Nagaraja V., Stieger M., Nager C., Hadi SM., Bickle T.A. (1985a) The nucleotide sequence recognised by the *Escherichia coli* D type I restriction and modification enzyme. *Nucleic Acids Res.* **13**: 389-399.

Nagaraja V., Shepherd J.C., Pripfl T., Bickle T.A. (1985b) Two type I restriction enzymes from *Salmonella* species. Purification and DNA recognition sequences. *J. Mol. Biol.* **182**: 579-587.

Nagaraja V., Shepherd J.C., Bickle T.A. (1985c) A hybrid recognition sequence in a recombinant restriction enzyme and the evolution of DNA sequence specificity. *Nature* **316**: 371-372.

Naito T., Kusano K., Kobayashi I. (1995) Selfish behaviour of restriction-modification systems. *Science* **267**: 897-899.

Nakayama Y., Kobayashi I. (1998) Restriction-modification gene complexes as selfish gene entities: roles of a regulatory system in their establishment, maintenance, and apoptotic mutual exclusion. *Proc. Natl. Acad. Sci. USA* **95**: 6442-6447.

Noyer-Weidner M., Diaz R., Reiners L. (1986) Cytosine-specific DNA modification interferes with plasmid establishment in *Escherichia coli* K12: involvement of *rglB*. *Mol. Gen. Genet.* **205**: 469-475.

Ogura T., Inoue K., Tatsuta T., Suzaki T., Karata K., Young K., Su L.H., Fierke C.A., Jackman J.E., Raetz C.R., Coleman J., Tomoyasu T., Matsuzawa H. (1999) Balanced biosynthesis of major membrane components through regulated degradation of the committed enzyme of lipid A biosynthesis by the AAA protease FtsH (HflB) in *Escherichia coli*. *Mol. Microbiol.* **31**: 833-844.

O'Neill M., Chen A., Murray N.E. (1997) The restriction-modification genes of *Escherichia coli* K-12 may not be selfish: they do not resist loss and are readily replaced by alleles conferring different specificities. *Proc. Natl. Acad. Sci. USA* **94**: 14596-14601.

O'Neill M., Dryden D.T., Murray N.E. (1998) Localization of a protein-DNA interface by random mutagenesis. *EMBO J.* **17**: 7118-7127.

Opperman T., Murli S., Smith B.T., Walker G.C. (1999) A model for a *umuDC*-dependent prokaryotic DNA damage checkpoint. *Proc. Natl. Acad. Sci. USA* **96**: 9218-9223.

Osborn M., Person S., Phillips S., Funk F. (1967) A determination of mutagen specificity in bacteria using nonsense mutants of bacteriophage T4. *J. Mol. Biol.* **26**: 437-447.

Pak M., Wickner S. (1997) Mechanism of protein remodeling by ClpA chaperone. *Proc. Natl. Acad. Sci. USA* **94**: 4901-4906.

Panne D., Raleigh E.A., Bickle T.A. (1998) McrBs, a modulator peptide for McrBC activity. *EMBO J.* **17**: 5477-5483.

Panne D., Raleigh E.A., Bickle T.A. (1999) The McrBC endonuclease translocates DNA in a reaction dependent on GTP hydrolysis. *J. Mol. Biol.* **290**: 49-60.

Pein C.D., Reuter M., Cech D., Kruger D.H. (1989) Oligonucleotide duplexes containing CC(A/T)GG stimulate cleavage of refractory DNA by restriction endonuclease EcoRII. *FEBS Lett.* **245**: 141-144.

Peterson K.R., Wertman K.F., Mount D.W., Marinus M.G. (1985) Viability of *Escherichia coli* K-12 DNA adenine methylase (*dam*) mutants requires increased expression of specific genes in the SOS regulon. *Mol. Gen. Genet.* **201**: 14-19.

Peterson K.R., Mount D.W. (1993) Analysis of the genetic requirements for viability of *Escherichia coli* K-12 DNA adenine methylase (*dam*) mutants. *J. Bacteriol.* **175**: 7505-7508.

Phillips T.A., VanBogelen R.A., Neidhardt F.C. (1984) *lon* gene product of *Escherichia coli* is a heat-shock protein. *J. Bacteriol.* **159**: 283-287.

Piekarowicz A., Bickle T.A., Shepherd J.C., Ineichen K. (1981) The DNA sequence recognised by the *Hinf*III restriction endonuclease. *J. Mol. Biol.* **146**: 167-172.

Pieper U., Brinkmann T., Kruger T., Noyer-Weidner M., Pingoud A. (1997) Characterization of the interaction between the restriction endonuclease McrBC from *E. coli* and its cofactor GTP. *J. Mol. Biol.* **272**: 190-199.

Posfai J., Bhagwat A.S., Posfai G., Roberts R.J. (1989) Predictive motifs derived from cytosine methyltransferases. *Nucleic Acids Res.* **17**: 2421-2435.

Powell L.M., Dryden D.T., Murray N.E. (1998) Sequence-specific DNA binding by *Eco*KI, a type IA DNA restriction enzyme. *J. Mol. Biol.* **283**: 963-976.

Prakash-Cheng A., Ryu J. (1993) Delayed expression of in vivo restriction activity following conjugal transfer of *Escherichia coli hsdK* (restriction-modification) genes. *J. Bacteriol.* **175**: 4905-4906.

Prakash-Cheng A., Chung S.S., Ryu J. (1993) The expression and regulation of *hsdK* genes after conjugative transfer. *Mol. Gen. Genet.* **241**: 491-496.

Pratt L.A., Silhavy T.J. (1996) The response regulator SprE controls the stability of RpoS. *Proc. Natl. Acad. Sci. USA* **93**: 2488-2492.

Price C., Pripfl T., Bickle T.A. (1987) *Eco*R124 and *Eco*R124/3: the first members of a new family of type I restriction and modification systems. *Eur. J. Biochem.* **167**: 111-115.

Pukkila P.J., Peterson J., Herman G., Modrich P., Meselson M. (1983) Effects of high levels of DNA adenine methylation on methyl-directed mismatch repair in *Escherichia coli. Genetics* **104**: 571-582.

Radman M. (1975) SOS repair hypothesis: phenomenology of an inducible DNA repair which is accompanied by mutagenesis. *Basic Life Sci.* **5**: 355-367.

Rajagopalan M., Lu C., Woodgate R., O'Donnell M., Goodman M.F., Echols H. (1992) Activity of the purified mutagenesis proteins UmuC, UmuD', and RecA in replicative bypass of an abasic DNA lesion by DNA polymerase III. *Proc. Natl. Acad. Sci. USA* **89**: 10777-10781.

Raleigh E.A., Wilson G. (1986) *Escherichia coli* K-12 restricts DNA containing 5-methylcytosine. *Proc. Natl. Acad. Sci. USA* **83**: 9070-9074.

Raleigh E.A., Murray N.E., Revel H., Blumenthal R.M., Westaway D., Reith A.D., Rigby P.W., Elhai J., Hanahan D. (1988) McrA and McrB restriction phenotypes of some *E. coli* strains and implications for gene cloning. *Nucleic Acids Res.* **16**: 1563-1575.

Raleigh E.A., Trimarchi R., Revel H. (1989) Genetic and physical mapping of the *mcrA* (*rglA*) and *mcrB* (*rglB*) loci of *Escherichia coli* K-12. *Genetics* **122**: 279-296.

Raleigh E.A., Benner J., Bloom F., Braymer H.D., DeCruz E., Dharmalingam K., Heitman J., Noyer Weidner M., Piekarowicz A., Kretz P.L., *et al.* (1991) Nomenclature relating to restriction of modified DNA in *Escherichia coli. J. Bacteriol.* **173**: 2707-2709.

Rao D.N., Eberle H., Bickle T.A. (1989) Characterization of mutations of the bacteriophage P1 *mod* gene encoding the recognition subunit of the *Eco*P1 restriction and modification system. *J. Bacteriol.* **171**: 2347-2352.

Rastorguev S.M., Zavilgelsky G.B., Tchurikov N.A. (1998) IncI1 plasmid R64 encodes the ArsR protein that alleviates type I restriction. *FEBS Lett.* **426**: 21-23.

Rasulova F.S., Dergousova N.I., Starkova N.N., Melnikov E.E., Rumsh L.D., Ginodman L.M., Rotanova T.V. (1998) The isolated proteolytic domain of *Escherichia coli* ATP-dependent protease Lon exhibits the peptidase activity. *FEBS Lett.* **432**: 179-181.

Ravetch J.V., Horiuchi K., Zinder N.D. (1978) Nucleotide sequence of the recognition site for the restriction-modification enzyme of *Escherichia coli* B. *Proc. Natl. Acad. Sci. USA* **75**: 2266-2270.

Ravi R.S., Sozhamannan S., Dharmalingam K. (1985) Transposon mutagenesis and genetic mapping of the *rglA* and *rglB* loci of *Escherichia coli. Mol. Gen. Genet.* **198**: 390-392.

Read T.D., Thomas A.T., Wilkins B.M. (1992) Evasion of type I and type II DNA restriction systems by IncI1 plasmid CoIIb-P9 during transfer by bacterial conjugation. *Mol. Microbiol.* **6**: 1933-1941.

Redaschi N., Bickle T.A. (1996) Posttranscriptional regulation of *Eco*P1I and *Eco*P15I restriction activity. *J. Mol. Biol.* **257**: 790-803.

Reiser J., Yuan R. (1977) Purification and properties of the P15 specific restriction endonuclease from *Escherichia coli. J. Biol. Chem.* **252**: 451-456.

Rimseliene R., Vaisvila R., Janulaitis A. (1995) The *eco72IC* gene specifies a trans-acting factor which influences expression of both DNA methyltransferase and endonuclease from the *Eco*72I restriction-modification system. *Gene* **157**: 217-219.

Roberts R.J. (1998) A new world of restriction and modification enzymes. *The NEB Transcript* **9**: 1-4.

Rogan E.G., Bessman M.J. (1970) Studies on the pathway of incorporation of 2-aminopurine into the deoxyribonucleic acid of *Escherichia coli. J. Bacteriol.* **103**: 622-633.

Rohrwild M., Coux O., Huang H.C., Moerschell R.P., Yoo S.J., Seol J.H., Chung C.H., Goldberg A.L. (1996) HslV-HslU: A novel ATP-dependent protease complex in *Escherichia coli* related to the eukaryotic proteasome. *Proc. Natl. Acad. Sci. USA* **93**: 5808-13.

Rohrwild M., Pfeifer G., Santarius U., Muller S.A., Huang H.-C., Engel A., Baumeister W., Goldberg A.L. (1997) The ATP-dependent HslVU protease from *Escherichia coli* is a four-ring structure resembling the proteasome. *Nature Struct. Biol.* **4**: 133-139.

Ronen A. (1980) 2-Aminopurine. *Mutat. Res.* **75**: 1-47.

Ross T.K., Achberger E.C., Braymer H.D. (1987) Characterization of the *Escherichia coli* modified cytosine restriction (*mcrB*) gene. *Gene* **61**: 277-289.

Ross T.K., Achberger E.C., Braymer H.D. (1989a) Nucleotide sequence of the McrB region of *Escherichia coli* K-12 and evidence for two independent translational initiation sites at the *mcrB* locus. *J. Bacteriol.* **171**: 1974-1981.

Ross T.K., Achberger E.C., Braymer H.D. (1989b) Identification of a second polypeptide required for McrB restriction of 5-methylcytosine-containing DNA in *Escherichia coli* K12. *Mol. Gen. Genet.* **216**: 402-407.

Rubin R.A., Modrich P. (1977) *Eco*RI methylase. Physical and catalytic properties of the homogeneous enzyme. *J. Biol. Chem.* **252**: 7265-7272.

Sain B., Murray N.E. (1980) The *hsd* (host specificity) genes of *E. coli* K-12. *Mol. Gen. Genet.* **180**: 35-46.

Saha S., Rao D.N. (1995) ATP hydrolysis is required for DNA cleavage by *Eco*PI restriction enzyme. *J. Mol. Biol.* **247**: 559-567.

Salles B., Defais M. (1984) Signal of induction of *recA* protein in *E. coli. Mutat. Res.* **131**: 53-59.

Schweder T., Lee K.H., Lomovskaya O., Matin A. (1996) Regulation of *Escherichia coli* starvation sigma factor (sigma s) by ClpXP protease. *J. Bacteriol.* **178**: 470-476.

Sektas M., Kaczorowski T., Podhajska A.J. (1992) Purification and properties of the *Mbo*II, a class-IIS restriction endonuclease. *Nucleic Acids Res.* **20**: 433-438.

Sektas M., Kaczorowski T., Podhajska A.J. (1995) Interaction of the *Mbo*II restriction endonuclease with DNA. *Gene* **157**: 181-185.

Semerjian A.V., Malloy D.C., Poteete A.R. (1989) Genetic structure of the bacteriophage P22 PL operon. *J. Mol. Biol.* **207**: 1-13.

Seol J.H., Woo K.M., Kang M.S., Ha D.B., Chung C.H. (1995) Requirement of ATP hydrolysis for assembly of ClpA/ClpP complex, the ATP-dependent protease Ti in *Escherichia coli. Biochem. Biophys. Res. Commun.* **217**: 41-51.

Seong I.S., Oh J.Y., Yoo S.J., Seol J.H., Chung C.H. (1999) ATP-dependent degradation of SulA, a cell division inhibitor, by the HslVU protease in *Escherichia coli. FEBS Lett.* **456**: 211-214.

Sharrocks A.D., Hornby D.P. (1991) Transcriptional analysis of the restriction and modification genes of bacteriophage P1. *Mol. Microbiol.* **5**: 685-694.

Shinagawa H., Iwasaki H., Kato T., Nakata A. (1988) RecA protein-dependent cleavage of UmuD protein and SOS mutagenesis. *Proc. Natl. Acad. Sci. USA* **85**: 1806-1810.

Shotland Y., Koby S., Teff D., Mansur N., Oren D.A., Tatematsu K., Tomoyasu T., Kessel M., Bukau B., Ogura T., Oppenheim A.B. (1997) Proteolysis of the phage lambda CII regulatory protein by FtsH (HflB) of *Escherichia coli*. *Mol. Microbiol.* **24**: 1303-1310.

Simmon V.F., Lederberg S. (1972) Degradation of bacteriophage lambda deoxyribonucleic acid after restriction by *Escherichia coli* K-12. *J. Bacteriol.* **112**: 161-169.

Singer M., Baker T.A. Schnitzler G., Deischel S.M., Goel M., Dove W., Jaacks K.J., Grossman A.D., Erickson J.W., Gross C.A. (1989) A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol. Rev.* **53**: 1-24.

Snyder M., Drlica K. (1979) DNA gyrase on the bacterial chromosome: DNA cleavage induced by oxolinic acid. *J. Mol. Biol.* **131**: 287-302.

Spoerel N., Herrlich P., Bickle T.A. (1979) A novel bacteriophage defence mechanism: the anti-restriction protein. *Nature* **278**: 30-34.

Squires C.L., Pedersen S., Ross B.M., Squires C. (1991) ClpB is the *Escherichia coli* heat shock protein F84.1. *J. Bacteriol.* **173**: 4254-4262.

Smith C.K., Baker T.A., Sauer R.T. (1999) Lon and Clp family proteases and chaperones share homologous substrate-recognition domains. *Proc. Natl. Acad. Sci. USA* **96**: 6678-6682.

Smith J.D., Arber W., Kuhnlein U. (1972) Host specificity of DNA produced by *Escherichia coli*. XIV. The role of nucleotide methylation in *in vivo* B-specific modification. *J. Mol. Biol.* **63**: 1-8.

Stewart F.J., Raleigh E.A. (1998) Dependence of McrBC cleavage on distance between recognition elements. *Biol. Chem.* **379**: 611-616.

Streeck R.E. (1980) Single-strand and double-strand cleavage at half-modified and fully modified recognition sites for the restriction nucleases *Sau*3a and *Taq*I. *Gene* **12**: 267-275.

Streiff M.B., Iida S., Bickle T.A. (1987) Expression and proteolytic processing of the *darA* antirestriction gene product of bacteriophage P1. *Virology* **157**: 167-171.

Studier F.W., Movva N.R. (1976) SAMase gene of bacteriophage T3 is responsible for overcoming host restriction. *J. Virol.* **19**: 136-145.

Studier F.W., Bandyopadhyay P.K. (1988) Model for how type I restriction enzymes select cleavage sites in DNA. *Proc. Natl. Acad. Sci. USA* **85**: 4677-4681.

Sugino A., Peebles C.L., Kreuzer K.N., Cozzarelli N.R. (1977) Mechanism of action of nalidixic acid: purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. *Proc. Natl. Acad. Sci. USA* **74**: 4767-4771.

Suri B., Shepherd J.C., Bickle T.A. (1984) The *Eco*A restriction and modification system of *Escherichia coli* 15T-: enzyme structure and DNA recognition sequence. *EMBO J.* **3**: 575-579.

Suri B., Bickle T.A. (1985) *Eco*A: the first member of a new family of type I restriction modification systems. Gene organization and enzymatic activities. *J. Mol. Biol.* **186**: 77-85.

Sutherland E., Coe L., Raleigh E.A. (1992) McrBC: a multisubunit GTP-dependent restriction endonuclease. *J. Mol. Biol.* **225**: 327-348.

Tamura T., Nagy I., Lupas A., Lottspeich F., Cejka Z., Schoofs G., Tanaka K., De Mot R., Baumeister W. (1995) The first characterization of a eubacterial proteasome: the 20S complex of *Rhodococcus. Curr. Biol.* **5**: 766-74.

Tang M., Shen X., Frank E.G., O'Donnell M., Woodgate R., Goodman M.F. (1999) UmuD'(2)C is an error-prone DNA polymerase, *Escherichia coli* pol V. *Proc. Natl. Acad. Sci. USA* **96**: 8919-9824.

Tao T., Bourne J.C., Blumenthal R.M. (1991) A family of regulatory genes associated with type II restriction-modification systems. *J. Bacteriol.* **173**: 1367-1375.

Tao T., Blumenthal R.M. (1992) Sequence and characterization of *pvuIIR*, the *Pvu*II endonuclease gene, and of *pvuIIC*, its regulatory gene. *J. Bacteriol.* **174**: 3395-3398.

Taylor I., Patel J., Firman K., Kneale G. (1992) Purification and biochemical characterisation of the *Eco*R124 type I modification methylase. *Nucleic Acids Res.* **20**: 179-186.

Taylor I., Watts D., Kneale G. (1993) Substrate recognition and selectivity in the type IC DNA modification methylase M.*Eco*R124I. *Nucleic Acids Res.* **21**: 4929-4935.

Thoms B., Wackernagel W. (1982) UV-induced allevation of lambda restriction in *Escherichia coli* K-12: kinetics of induction and specificity of this SOS function. *Mol. Gen. Genet.* **186**: 111-117.

Thoms B., Wackernagel W. (1984) Genetic control of damage-inducible restriction alleviation in *Escherichia coli* K12: an SOS function not repressed by *lexA*. *Mol. Gen. Genet.* **197**: 297-303.

Thorpe P.H., Ternent D., Murray N.E. (1997) The specificity of *Sty*SKI, a type I restriction enzyme, implies a structure with rotational symmetry. *Nucleic Acids Res.* **25**: 1694-1700.

Titheradge A.J., Ternent D., Murray N.E. (1996) A third family of allelic hsd genes in *Salmonella enterica*: sequence comparisons with related proteins identify conserved regions implicated in restriction of DNA. *Mol. Microbiol.* **22**: 437-447.

Tomoyasu T., Yamanaka K., Murata K., Suzaki T., Bouloc P., Kato A., Niki H., Hiraga S., Ogura T. (1993) Topology and subcellular localization of FtsH protein in *Escherichia coli*. *J. Bacteriol.* **175**: 1352-1357.

Tomoyasu T., Gamer J., Bukau B., Kanemori M., Mori H., Rutman A.J., Oppenheim A.B., Yura T., Yamanaka K., Niki H., *et al.* (1995) *Escherichia coli* FtsH is a membrane-bound, ATP-dependent protease which degrades the heat-shock transcription factor sigma 32. *EMBO J.* **14**: 2551-2560.

Toothman P. (1980) Restriction alleviation by bacteriophages lambda and lambda reverse. *J. Virol.* **38**: 621-631.

Torres-Cabassa A.S., Gottesman S. (1987) Capsule synthesis in *Escherichia coli* K-12 is regulated by proteolysis. *J. Bacteriol.* **169**: 981-989.

Trisler P., Gottesman S. (1984) *lon* transcriptional regulation of genes necessary for capsular polysaccharide synthesis in *Escherichia coli* K-12. *J. Bacteriol.* **160**: 184-191.

Tucholski J., Skowron P.M., Podhajska A.J. (1995) *Mme*I, a class-IIS restriction endonuclease: purification and characterization. *Gene* **157**: 87-92.

Van Melderen L., Bernard P., Couturier M. (1994) Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria. *Mol. Microbiol.* **11**: 1151-1157.

Van Melderen L., Gottesman S. (1999) Substrate sequestration by a proteolytically inactive Lon mutant. *Proc. Natl. Acad. Sci. USA* **96**: 6064-6071.

Vertes A.A., Inui M., Kobayashi M., Kurusu Y., Yukawa H. (1993) Presence of *mrr*- and *mcr*-like restriction systems in coryneform bacteria. *Res. Microbiol.* **144**: 181-185.

Vovis G.F., Horiuchi K., Zinder N.D. (1974) Kinetics of methylation of DNA by a restriction endonuclease from *Escherichia coli* B. *Proc. Natl. Acad. Sci. USA* **71**: 3810-3813.

Wagner R. Jr., Meselson M. (1976) Repair tracts in mismatched DNA heteroduplexes. *Proc. Natl. Acad. Sci. USA* **73**: 4135-4139.

Wang J., Hartling J.A., Flanagan J.M. (1997) The structure of ClpP at 2.3 A resolution suggests a model for ATP-dependent proteolysis. *Cell* **91**: 447-456.

Wang R.F., O'Hara E.B., Aldea M., Bargmann C.I., Gromley H., Kushner S.R. (1998) *Escherichia coli mrsC* is an allele of *hflB*, encoding a membrane-associated ATPase and protease that is required for mRNA decay. *J. Bacteriol.* **180**: 1929-1938.

Wang T.C., Smith K.C. (1986) Inviability of *dam recA* and *dam recB* cells of *Escherichia coli* is correlated with their inability to repair DNA double-strand breaks produced by mismatch repair. *J. Bacteriol.* **165**: 1023-1025.

Ward C.B., Glaser D.A. (1969) Analysis of the chloramphenicol-sensitive and chloramphenicol-resistant steps in the initiation of DNA synthesis in *E. coli* B-r. *Proc. Natl. Acad. Sci. USA* **64**: 905-912.

Wawrzynow A., Wojtkowiak D., Marszalek J., Banecki B., Jonsen M., Graves B., Georgopoulos C., Zylicz M. (1995) The ClpX heat-shock protein of *Escherichia coli*, the ATP-dependent substrate specificity component of the ClpP-ClpX protease, is a novel molecular chaperone. *EMBO J.* **14**: 1867-1877.

Webb J.L., King G., Ternent D., Titheradge A.J., Murray N.E. (1996) Restriction by *Eco*KI is enhanced by co-operative interactions between target sequences and is dependent on DEAD box motifs. *EMBO J.* **15**: 2003-2009.

Wegrzyn A., Wegrzyn G., Taylor K. (1995) Protection of coliphage lambda O initiator protein from proteolysis in the assembly of the replication complex *in vivo*. *Virology* **207**: 179-184.

Weiserova M., Janscak P., Benada O., Hubacek J., Zinkevich V.E., Glover S.W., Firman K. (1993) Cloning, production and characterisation of wild type and mutant forms of the R.*Eco*K endonucleases. *Nucleic Acids Res.* **21**: 373-379.

Wickner S., Gottesman S., Skowyra D., Hoskins J., McKenney K., Maurizi M.R. (1994) A molecular chaperone, ClpA, functions like DnaK and DnaJ. *Proc. Natl. Acad. Sci. USA* **91**: 12218-12222.

Willcock D.F., Dryden D.T., Murray N.E. (1994) A mutational analysis of the two motifs common to adenine methyltransferases. *EMBO J.* **13**: 3902-3908.

Wilson G.G., Murray N.E. (1991) Restriction and modification systems. *Annu. Rev. Genet.* **25**: 585-627.

Winkler F.K., Banner D.W., Oefner C., Tsernoglou D., Brown R.S., Heathman S.P., Bryan R.K., Martin P.D., Petratos K., Wilson K.S. (1993) The crystal structure of

*Eco*RV endonuclease and of its complexes with cognate and non-cognate DNA fragments. *EMBO J.* **12**: 1781-1795.

Wojtkowiak D., Georgopoulos C., Zylicz M. (1993) Isolation and characterization of ClpX, a new ATP-dependent specificity component of the Clp protease of *Escherichia coli. J. Biol. Chem.* **268**: 22609-22617.

Woo K.M., Kim K.I., Goldberg A.L., Ha D.B., Chung C.H. (1992) The heat-shock protein ClpB in *Escherichia coli* is a protein-activated ATPase. *J. Biol. Chem.* **267**: 20429-20434.

Wood W.D. (1966) Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affecting the restriction and modification of DNA. *J. Mol. Biol.* **16**: 118-133.

Woodcock D.M., Crowther P.J., Diver W.P., Graham M., Bateman C., Baker D.J., Smith S.S. (1988) RglB facilitated cloning of highly methylated eukaryotic DNA: the human L1 transposon, plant DNA, and DNA methylated in vitro with human DNA methyltransferase. *Nucleic Acids Res.* **16**: 4465-4482.

Woodcock D.M., Crowther P.J., Doherty J., Jefferson S., DeCruz E., Noyer-Weidner M., Smith S.S., Michael M.Z., Graham M.W. (1989) Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res.* **17**: 3469-3478.

Wu W.F., Zhou Y., Gottesman S. (1999) Redundant *in vivo* proteolytic activities of *Escherichia coli* Lon and the ClpYQ (HslUV) protease. *J. Bacteriol.* **181**: 3681-3687.

Xu G., Willert J., Kapfer W., Trautner T.A. (1995) *Bsu*CI, a type-I restriction-modification system in *Bacillus subtilis. Gene* **157**: 59.

Yoo O.J., Agarwal K.L. (1980) Isolation and characterization of two proteins possessing Hpa II methylase activity. *J. Biol. Chem.* **255**: 6445-6449.

Yoshimori R., Roulland-Dussoix D., Boyer H.W. (1972) R factor-controlled restriction and modification of deoxyribonucleic acid: restriction mutants. *J. Bacteriol.* **112**: 1275-1279.

Yuan R., Meselson M. (1970) A specific complex between a restriction endonuclease and its DNA substrate. *Proc. Natl. Acad. Sci. U S A* **65**: 357-362.

Yuan R., Hamilton D.L., Burckhardt J. (1980) DNA translocation by the restriction enzyme from *E. coli* K. *Cell* **20**: 237-244.

Zabeau M., Schell J., Van Montagu M. (1973) The alleviation of host-controlled restriction of unmodified phages by functions of bacteriophage lambda. *Arch. Int. Physiol. Biochim.* **81**: 990.

Zabeau M., Friedman S., Van Montagu M., Schell J. (1980) The *ral* gene of phage lambda. I. Identification of a non-essential gene that modulates restriction and modification in *E. coli. Mol. Gen. Genet.* **179**: 63-73.

Zissler J., Sinder E.R., Schaefer F. (1971) The role of recombination in growth of bacteriophage lambda. In *The bacteriophage lambda.* A.D.Hershey (ed), Cold Spring Harbor Laboratories, New York, pp455-475.

Zylicz M., Liberek K., Wawrzynow A., Georgopoulos C. (1998) Formation of the preprimosome protects lambda O from RNA transcription-dependent proteolysis by ClpP/ClpX. *Proc. Natl. Acad. Sci. USA* **95**: 15259-15263.

Molecular Microbiology (1998) **28**(1), 25–35

ClpX and ClpP are essential for the efficient acquisition of genes specifying type IA and IB restriction systems

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Summary

Efficient acquisition of genes that encode a restriction and modification (R–M) system with specificities different from any already present in the recipient bacterium requires the sequential production of the new modification enzyme followed by the restriction activity in order that the chromosome of the recipient bacterium is protected against attack by the restriction endonuclease. We show that ClpX and ClpP, the components of ClpXP protease, are necessary for the efficient transmission of the genes encoding *Eco*KI and *Eco*AI, representatives of two families of type I R–M systems, thus implicating ClpXP in the modulation of restriction activity. Loss of ClpX imposed a bigger barrier than loss of ClpP, consistent with a dual role for ClpX, possibly as a chaperone and as a component of the ClpXP protease. Transmission of genes specifying *Eco*KI was more dependent on ClpX and ClpP than transmission of the genes for *Eco*AI. Sensitivity to absence of the protease was also influenced by the mode of gene transfer; conjugative transfer and transformation were more dependent on ClpXP than transduction. In the absence of either ClpX or ClpP transfer of the *Eco*KI genes by P1-mediated transduction was impaired, transfer of the *Eco*AI genes was not.

Introduction

There are two regions within the chromosome of *Escherichia coli* where effective recombinational replacement produces hypervariable, or polymorphic, loci (Milkman, 1997). One includes the O-antigen gene complex at 45 min (see Hobbs and Reeves, 1994) and the other includes the restriction and modification (R–M) genes at 98.5 min (see Barcus and Murray, 1995). In *E. coli* and *Salmonella enterica*, the latter region is already known to specify as many as 16 alternative type I R–M systems, each conferring a different sequence specificity. The efficient acquisition of R–M genes conferring specificities different from that of the recipient requires the sequential production of the new modification and restriction activities, and the consequent protection of the recipient chromosome against attack by the restriction endonuclease. Failure to modify the targets in the recipient chromosome, before the production of the restriction enzyme, would result in cell death. This paper identifies two genes of *E. coli* K-12 that are essential for the efficient transmission of chromosomal genes encoding type I R–M systems; the products of these genes may modulate restriction activity.

At least three families of type I R–M systems are encoded by alleles within the hypervariable locus in *E. coli* (Barcus *et al.*, 1995), although members of only two of these (IA and IB) have been well documented. In this paper, the transmission of genes representing both of these families is investigated.

Three genes, *hsd* (for host specificity of DNA) *R*, *M* and *S*, encode the subunits of each type I R–M system. All three subunits combine to make a large oligomeric protein that, in response to the pattern of adenine methylation within specific DNA sequences, functions to maintain the methylation pattern of the resident DNA and to cut foreign DNA that is devoid of methylated bases within the specific target sequences. HsdS confers sequence specificity to both the modification and restriction activities. For *Eco*KI, the R–M system found in *E. coli* K-12, the stoichiometry of the subunits is 2HsdR, 2HsdM and 1HsdS (Dryden *et al.*, 1997), commonly abbreviated to $R_2M_2S_1$, and this complex coexists *in vivo* with a smaller one, M_2S_1, endowed with only modification activity (Dryden *et al.*, 1993). A similar relationship is expected for *Eco*AI.

The *hsd* genes of *E. coli* K-12 have two promoters (Loenen *et al.*, 1987), one (*pmod*) for the transcription of *hsdM* and *S* and the consequent production of the modification enzyme, the other (*pres*) for transcription of *hsdR* to provide HsdR(R), which on association with the modification enzyme generates the restriction endonuclease ($R_2M_2S_1$). Experiments have failed to find evidence that transcriptional control of the *hsd* genes accounts for the sequential production of the modification and restriction activities. Transcription from *pres* is unaffected by the modification phenotype of the bacteria (Loenen *et al.*, 1987) and transcription

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from *pmod* and *pres* occurs simultaneously after conjugative transfer to a modification-deficient recipient (Prakash-Cheng *et al.*, 1993). Other experiments have shown a lag of many generations before transconjugants become restriction proficient, despite transcription of the *hsd* genes (Prakash-Cheng and Ryu, 1993). Post-translational control, possibly at the level of subunit assembly, could be a critical factor in causing a delay in the production of the restriction enzyme, and Dryden *et al.* (1997) have used their data from an *in vitro* assembly pathway as the basis of such a mechanism. In the assembly pathway they propose, both inactive intermediates in the assembly pathway and the HsdR polypeptide could be susceptible to proteases. A mutation isolated in *E. coli* C, which results in cell death after the acquisition of *hsd* genes (Prakash-Cheng *et al.*, 1993), has identified a gene, *hsdC*, the product of which is a candidate for influencing the assembly pathway of *Eco*KI (Dryden *et al.*, 1997).

In this paper, we screen *E. coli* K-12 mutants deficient in proteases for their ability to acquire R–M genes. We include those mutants currently shown to lack ATP-dependent proteases (*clpP*, *A*, *X*, *Q*, *Y*, *hflA* and *lon*) and a mutant now known to have low amounts of the protease encoded by *hflB*; this gene, also known as *ftsH*, is an essential gene and it is therefore impossible to use null mutations (see Kihara *et al.*, 1997; Shotland *et al.*, 1997). We find that the products of both *clpP* and *clpX* affect the efficient transmission of *hsd* genes by conjugation, transformation and transduction, although they do not affect members of the two families in the same way.

Results

Do proteases affect the introduction of type I R–M genes by conjugation?

The allelic *hsd* genes that encode *Eco*KI and *Eco*AI, the best studied representatives of two families of type I R–M systems, can be transferred by conjugation to recipients lacking appropriate modification of their DNA without any detectable killing of the potentially vulnerable cells. *E. coli* C is a natural isolate of *E. coli* that lacks *hsd* genes and is commonly used as a restriction- and modification-deficient (r^-m^-) strain. A derivative of *E. coli* C (*hsdC*) has been isolated that, unlike the wild type, dies after the receipt of the *hsd* genes encoding either *Eco*KI (Prakash-Cheng *et al.*, 1993) or *Eco*AI (Kulik and Bickle, 1996).

It has been suggested that when *hsd* genes enter a recipient cell proteases could contribute to a post-transcriptional mechanism for ensuring the sequential production of the modification methylase before the restriction endonuclease (Kulik and Bickle, 1996; Dryden *et al.*, 1997; J. Ryu, personal communication). As a test of this idea, a series of $r_K^-m_K^-$ recipients of *E. coli* K-12 was made in which each strain was defective in a structural component of a known protease, and these strains were monitored for the efficiency with which they acquired F′ plasmids carrying functional *hsd* genes. The donors of the F′ plasmids were *recA* to prevent recombination between the F′ and the donor chromosome as recombination could in some cases result in early conjugative transfer of the wild-type allele of the protease gene to the recipient. Pairs of F′ plasmids, one $hsdR^+M^+S^+$ and the other $hsdR^-M^+S^+$, were used for both the *Eco*KI and *Eco*AI systems. The titres of recipients and of conjugants acquiring a selectable plasmid marker (Tc^r or Cm^r, see Tables 1 and 2) were determined after 2.5 h. When the plasmid conferred an r^+ phenotype, the titres of both conjugants and recipients that survived conjugation were severely depressed for two of the eight *E. coli* K-12 mutants, *clpX* and *clpP*, as well as for the *hsdC* strain used as a positive control (see Table 1 for *Eco*KI and Table 2 for *Eco*AI). For both families of systems, therefore, *clpP* and *clpX* are implicated as relevant to the sequential establishment of the modification and restriction activities.

In the case of *clpP*, *clpX* and *hsdC*, in which the mutations imposed a barrier to the acquisition of the drug resistance marker present on the F′, a sample of transconjugants was tested for restriction and modification. Similar checks were made on progeny from the clp^+ and $hsdC^+$ control experiments. For *Eco*KI, r_K^+ derivatives were rare among the survivors of either the *clpX* recipient (2 out of 100) or the *hsdC* strain (0 out of 57). In contrast, half (10 out of 20) of the *clpP* transconjugants were r_K^+ and all the transconjugants tested from the negative control experiments (NM840 and JR300 *gyrA*) were restriction proficient. The presence of the F′ plasmid in the $r_K^-m_K^-$ transconjugants was confirmed by their sensitivity to the male-specific phage, M13, and by their ability to transfer Tc^r by conjugation. The $r_K^-m_K^-$ transconjugants behaved as if they had mutations in the *hsd* genes of the F′ plasmid; they could transfer an F′, but the plasmid no longer conferred an r_K^+ phenotype to the recipient cell. The barrier against the *Eco*AI system was less effective, irrespective of the genotype of the recipient, and most (19 out of 20) of the conjugants displayed the r_A^+ phenotype. Therefore, the titres of Tc^r conjugants (Table 2) closely reflect the titres of r_A^+ conjugants. In contrast, for *clpP*, *clpX* and *hsdC*, the titres of Tc^r conjugants overestimate the titres of r_K^+ conjugants; the corrected figures are given in the penultimate column (Table 1).

The low titre of recipients obtained when *clpX*, *clpP* and *hsdC* strains receive an hsd^+, rather than an $hsdR^-M^+S^+$ plasmid, is consistent with cell death after the premature production of the restriction enzyme (Table 1). The kinetics of the killing effect, following transfer of the hsd_K^+ plasmid to various recipient strains is shown in Fig. 1A. No killing was detected if the F′ plasmid had a defect in *hsdR* (Fig. 1B).

The products of *clpX* and *clpP* together make a complex

Table 1. Conjugative transfer of hsd_K genes to r^-m^- recipients.

Recipient strains	Donor strains: JC9935 F′ $r_K^-m_K^+$[a] Titre of recipients[b] after conjugation (ml^{-1})	JC9935 F′ $r_K^-m_K^+$[a] Titre of Tc^r (Cm^r) conjugants[b,c] (ml^{-1})	JC9935 F′ $r_K^+m_K^+$[a] Titre of recipients[b] after confugration (ml^{-1})	JC9935 F′ $r_K^+m_K^+$[a] Titre of Tc^r (Cm^r) conjugants[b,c] (ml^{-1})	JC9935 F′ $r_K^+m_K^+$[a] Titre of Tc^r (Cm^r) r^+m^+ conjugants (ml^{-1})	Relative frequency (%) of survival of recipients[d]
NM840 (C600 *Δhsd gyrA*)	2.1×10^8	2.1×10^8	2.1×10^8	2.1×10^8	2.1×10^8	100.0
NM840 *clpP*	1.6×10^8	1.6×10^8	1.8×10^5	7.4×10^4	3.7×10^4	0.11
NM840 *clpX*	1.4×10^8	1.4×10^8	3.5×10^4	4.8×10^3	9.6×10^1	0.03
NM840 *clpA*	1.6×10^8	1.6×10^8	1.6×10^8	1.4×10^8	NT	100.0
NM840 *clpQ*	9.9×10^7	9.8×10^7	9.7×10^7	9.7×10^7	NT	98.0
NM840 *clpY*	6.9×10^7	6.8×10^7	8.5×10^7	8.5×10^7	NT	123.2
NM840 *lon*	5.2×10^7	4.5×10^7	6.7×10^7	4.8×10^7	NT	128.8
NM840 *hflA*	7.1×10^7	3.2×10^7	1.0×10^8	9.5×10^7	NT	140.8
NM840 *hflB*	6.2×10^7	2.5×10^7	6.2×10^7	4.4×10^7	NT	100.0
JR300 *gyrA* (*E. coli* C)	1.5×10^8	1.5×10^8	1.5×10^8	1.5×10^8	1.5×10^8	100.0
NM820 (*E. coli* C *hsdC*)	2.0×10^8	2.0×10^8	2.6×10^4	4.8×10^3	$<8.4\times10^1$	0.01

a. F′ $r_K^-m_K^+$ (F′101-202) and F′ $r_K^+m_K^+$ (F′101-201) are derivatives of F′101-102 and F′101-101, respectively (Prakash-Cheng *et al.*, 1993), with miniTn*5*-Cm insertions (de Lorenzo *et al.*, 1990). The former includes $hsd_KR^-M^+S^+$ the latter $hsd_KR^+M^+S^+$.
b. Nalidixic acid was used to select against the donor, with the exception of NM820 in which kanamycin was used.
c. Tetracycline was used to select for transconjugants. When the recipient was Tc^r (NM840 *lon*, NM840 *hflA* and NM840 *hflB*) chloramphenicol was used.
d. Frequency (%) with which recipient cells survived conjugation with the donor of F′101-201 is expressed relative to that for F′101-202 (the ratio of the titre of recipient cells after conjugation with JC9935 F′101-201 to the titre of recipient cells after conjugation with JC9935 F′101-202 as a percentage).
NT, Conjugants have not been tested for restriction modification.

that functions as a protease, ClpX itself is a chaperone (Levchenko *et al.*, 1995; Gottesman, 1996); either or both activities could be relevant to the delayed production of the restriction enzyme after transcription of the *hsd* genes. The finding that the effect of *clpX* is more severe than that of *clpP* supports a dual role for ClpX.

Does the ClpXP complex affect the acquisition of hsd *genes by transformation?*

Bacteria were made competent in the uptake of DNA and scored for the efficiency of uptake of plasmid DNA carrying *hsd* genes (Table 3). In each series of experiments,

Table 2. Conjugative transfer of hsd_A genes to r^-m^- recipients.

Recipient strains	Donor strains: JC9935 F′ $r_A^-m_A^+$[a] Titre of recipients[b] after conjugation (ml^{-1})	JC9935 F′ $r_A^-m_A^+$[a] Titre of Tc^r (Cm^r) conjugants[b,c] (ml^{-1})	JC9935 F′ $r_A^+m_A^+$[a] Titre of recipients[b] after confugration (ml^{-1})	JC9935 F′ $r_A^+m_A^+$[a] Titre of Tc^r (Cm^r) conjugants[b,c] (ml^{-1})	Relative frequency (%) of survival of recipients[d]
NM840 (C600 *Δhsd gyrA*)	6.0×10^7	5.9×10^7	6.0×10^7	6.0×10^7	100.0
NM840 *clpP*	3.3×10^7	3.1×10^7	6.2×10^5	1.6×10^5	1.9
NM840 *clpX*	5.1×10^7	4.0×10^7	1.3×10^5	2.8×10^4	0.3
NM840 *clpA*	1.4×10^8	1.1×10^8	1.5×10^8	1.0×10^8	107.1
NM840 *clpQ*	9.9×10^7	9.3×10^7	9.7×10^7	8.4×10^7	98.0
NM840 *clpY*	5.5×10^7	3.8×10^7	4.8×10^7	4.8×10^7	87.3
NM840 *lon*	3.8×10^7	3.8×10^7	4.3×10^7	3.2×10^7	113.2
NM840 *hflA*	4.3×10^7	4.2×10^7	4.9×10^7	4.6×10^7	114.0
NM840 *hflB*	5.1×10^7	3.3×10^7	4.2×10^7	3.9×10^7	82.4
JR300 *gyrA* (*E. coli* C)	1.7×10^8	1.7×10^8	1.0×10^8	7.1×10^7	58.8
NM820 (*E. coli* C *hsdC*)	1.8×10^8	1.7×10^8	9.1×10^6	5.5×10^6	5.1

a. F′ $r_A^-m_A^+$ (F′101-302) and F′ $r_A^+m_A^+$ (F′101-301) are derivatives of F′101 (Low, 1972) in which *Eco*KI genes were replaced by $hsd_AR^-M^+S^+$ and $hsd_AR^+M^+S^+$ respectively. Both plasmids have *zjj*::Tn*10* and miniTn*5*-Cm insertions as described in *Experimental procedures*.
b. Nalidixic acid was used to select against the donor, with the exception of NM820 in which kanamycin was used.
c. Tetracycline was used to select for transconjugants. When the recipient was Tc^r (NM840 *lon*, NM840 *hflA* and NM840 *hflB*) chloramphenicol was used.
d. Frequency (%) with which recipient cells survived conjugation with the donor of F′101-301 is expressed relative to that for F′101-302 (the ratio of the titre of recipient cells after conjugation with JC9935 F′101-301 to the titre of recipient cells after conjugation with JC9935 F′101-302 as a percentage).

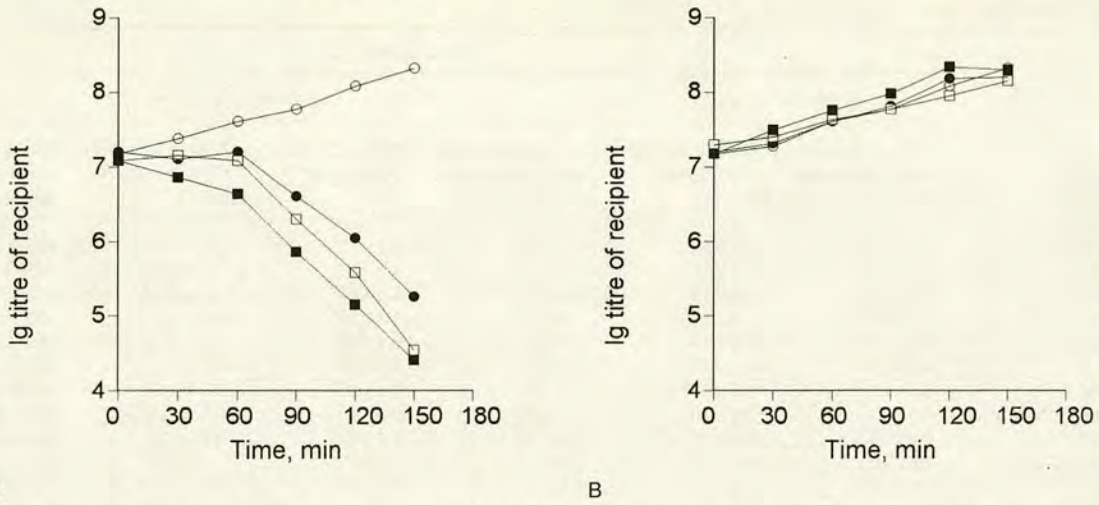

Fig. 1. Conjugative transfer of (A) $hsd_KR^+M^+S^+$ and (B) $hsd_KR^-M^+S^+$ genes to NM840 (○), NM840 *clpP* (●), NM840 *clpX* (□) and NM820 *E. coli* C *hsdC* (■). Cultures of donor (JC9935 F′101-201 and JC9935 F′101-202) and recipient bacteria, grown to mid-log phase, were mixed at a ratio of 10:1 incubated at 37°C and samples were plated at 30 min intervals on media selective for recipients (nalidixic acid for NM840, NM840 *clpP* and NM840 *clpX* and kanamycin for NM820).

a marker plasmid was mixed with the test plasmid. The marker was pBRK, a derivative of pBR322 in which the *bla* gene had been inactivated by the insertion of the *kan* gene from Tn*903* at the *Pst*I site. The test plasmid was a Tc^s Ap^r derivative of pBR322 including *hsd* genes, either $R^+M^+S^+$ or $R^-M^+S^+$. The ratios of Ap^r/Tc^r transformants were monitored; ratios lower than those obtained for clp^+ or $hsdC^+$ bacteria indicated a reduced efficiency in the recovery of the plasmid carrying the *hsd* genes. Significant variations were obtained only for plasmids including $hsdR^+$, M^+ and S^+. The *clpP*, *clpX* and *hsdC* mutations depressed the recovery of hsd^+ plasmids by transformation, although the effect on the plasmid encoding the *Eco*AI system was smaller than that on the one encoding *Eco*KI. For *Eco*KI particularly, the *hsdC* strain had a bigger barrier than either *clpX* or *clpP* derivatives of *E. coli* K-12. No transformant of the *hsdC* strain was obtained for the *hsd* plasmid carrying the *Eco*KI genes, whereas among the Ap^r transformants of *clpX* and *clpP* strains appreciable numbers (20% and 70%, respectively) were r_K^+.

The effect of clpP *and* clpX *on the transfer of* hsd *genes by P1 transduction*

The efficiency of co-transduction of *hsd* with *dnaC*, a closely linked marker, was assessed for $hsdR^+M^+S^+$ (r^+m^+) and $hsdR^-M^+S^+$ (r^-m^+) donors. The *dnaC325* mutation confers a temperature-sensitive lethal phenotype. P1 lysates made on hsd_K and hsd_A donors were used to transduce r^-m^- *dnaC* recipient strains. $DnaC^+$ transductants were selected at 42°C and scored for the acquisition of *hsd* genes and the loss of Tn*10*, a marker for Tc^r on the opposite side of *dnaC* from the *hsd* deletion (Tables 4 and 5). The *hsdC* mutation in *E. coli* C has been shown to prevent the co-transduction of the hsd^+ genes encoding *Eco*DI, like *Eco*KI a member of the type IA family, but not if the donor is $hsdR^-M^+S_D^+$ (O'Neill *et al.*, 1997), and for this reason the experiment using an *hsdC* host was omitted for *Eco*KI. The effect of *clpX* on *Eco*KI (Table 4) resembled that of *hsdC* on the acquisition of *Eco*DI (O'Neill *et al.*, 1997); *clpP* has a smaller effect than either *clpX* or *hsdC*. A very different result was obtained for the type IB

Table 3. The effect of *clpP* and *clpX* on uptake of *hsd* genes by transformation.

Recipient strains	Ratio of transformants with *hsd* plasmids to control plasmid			
	p*hsdRMS*$_K$ (p*hsd*$^+$)	p*hsdMS*$_K$ (p*hsdR*$^-$)	p*hsdRMS*$_A$ (pFFP30)	p*hsdMS*$_A$ (pFFP31)
NM840 (C600 Δ*hsd gyrA*)	3.0	10.9	9.5	6.9
NM840 *clpP*	6.7×10^{-2}	6.6	1.7	5.6
NM840 *clpX*	6.6×10^{-3}	10.3	2.3	7.3
JR300 (*E. coli* C)	2.4	6.8	4.3	5.8
NM820 (*E. coli* C *hsdC*)	$<10^{-4}$ [a]	5.3	6.0×10^{-1}	8.7

a. No transformants isolated.

Table 4. The effect of *clpP* and *clpX* on uptake of hsd_K genes by P1 transduction.

	Donor strains					
	C600 ($hsd_KR^+M^+S^+$ *dnaC*$^+$)			5K ($hsd_KR^-M^+S^+$ *dnaC*$^+$)		
Recipient strains	Sample size	*dnaC*$^+$–*hsd*$^+$ linkage	*dnaC*$^+$–Tn*10*° linkage	Sample size	*dnaC*$^+$–*hsdM*$^+S^+$ linkage	*dnaC*$^+$–Tn*10*° linkage
NK125 (NM840 *dnaC zjj*::Tn*10*)	50	0.32	0.90	50	0.42	1.00
NK125 *clpP*	100	0.09	0.96	100	0.31	0.96
NK125 *clpX*	100	0.00	0.94	50	0.46	0.98

representative; none of the three mutations had a demonstrable effect on the acquisition of the *hsd* genes of *Eco*AI (Table 5). In the tests based on either conjugation or transformation the barrier to the transmission of the *hsd* genes encoding *Eco*AI was weaker than that against *Eco*KI, nevertheless it was demonstrable even for *clpP*.

Do clpP *and* clpX *affect the level of restriction?*

It has been shown that increasing the number of copies of *hsdR* relative to *hsdM* and *S* decreases the efficiency of plating (e.o.p.) of unmodified phage λ (Webb *et al.*, 1996). It seems probable that increasing the number of copies of *hsdR* increases the concentration of the restriction endonuclease ($R_2M_2S_1$) relative to that of the modification methylase (M_2S_1). If the ClpXP protease normally modulates the level of restriction endonuclease, perhaps by attacking HsdR, then in its absence the balance between the levels of the endonuclease and the modification enzyme might be shifted in favour of the endonuclease.

The effect of the *clpP* and *clpX* mutations on the restriction of unmodified phage λ was assessed. Restriction by both *Eco*KI and *Eco*AI was increased in the absence of either ClpX or ClpP (Table 6). This is consistent with an elevated level of the active endonuclease in cells lacking the ClpXP protease. Similar elevated levels of restriction were also detected for *clpP* and *clpX* derivatives of AB1157, a strain of *E. coli* K-12 that already shows high levels of restriction because it lacks a cryptic prophage (Rac) including a gene that alleviates restriction (see Webb *et al.*, 1996). Elevated levels of restriction were not found for hsd^+_K derivatives of the *hsdC*$^-$ *E. coli* C (data not shown). Kulik and Bickle (1996) report that they fail to detect a change in restriction by *Eco*AI in an r_A^+ derivative of an *hsdC* derivative of *E. coli* C. This apparent difference between *E. coli* C and *E. coli* K-12 is not explicable at the present time.

Do clpP *and* clpX *affect the loss of* hsd *genes?*

Some type II R–M systems have been shown to stabilize the maintenance of the plasmid that encodes them (Kulakauskas *et al.*, 1995; Naito *et al.*, 1995). It has been argued that when R–M genes are lost and the restriction and modification enzymes cease to be made, the cells eventually lose the capacity to modify the many target sequences within their chromosomes and hence lose the capacity to protect themselves against residual restriction enzyme. Unmodified target sequences will elicit restriction and the cell will be killed. Some R–M systems therefore have been considered as 'plasmid addiction systems' (Naito *et al.*, 1995). *Eco*KI, however, does not behave in this way; genes encoding *Eco*KI are readily lost and replaced (O'Neill *et al.*, 1997). Nevertheless, if ClpXP affects the establishment and severity of the restriction-proficient phenotype, it might be anticipated that it could

Table 5. The effect of *clpP* and *clpX* on uptake of hsd_A genes by P1 transduction.

	Donor strains					
	WA2899 ($hsd_AR^+M^+S^+$ *dnaC*$^+$)			NM863 ($hsd_AR^-M^+S^+$ *dnaC*$^+$)		
Recipient strains	Sample size	*dnaC*$^+$–*hsd*$^+$ linkage	*dnaC*$^+$–Tn*10*° linkage	Sample size	*dnaC*$^+$–*hsdM*$^+S^+$ linkage	*dnaC*$^+$–Tn*10*° linkage
NK125 (NM840 *dnaC zjj*::Tn*10*)	60	0.48	0.83	50	0.54	0.90
NK125 *clpP*	100	0.63	0.88	49	0.51	0.81
NK125 *clpX*	100	0.65	0.80	49	0.63	0.84
NM824 (*E. coli* C *dnaC zjj*::Tn*10*)	48	0.31[a]	0.75	48	0.33[a]	0.83
NM822 (*E. coli* C *hsdC dnaC zjj*::Tn*10*)	50	0.42[a]	0.90	48	0.42[a]	0.90

a. Lower frequencies of co-transduction were observed in similar transduction experiments between *E. coli* K-12 and *E. coli* C (O'Neill *et al.*, 1997).

Table 6. The effect of *clpP* and *clpX* on expression of restriction activity.

Strains	Efficiency of plating (e.o.p.)[a]	
	λ*vir*.0	λ*vir*.K
*Eco*KI system[b]		
C600 *gyrA*	$(1.43 \pm 0.02) \times 10^{-4}$	0.75 ± 0.05
C600 *gyrA clpP*	$(2.03 \pm 0.85) \times 10^{-5}$	0.86 ± 0.29
C600 *gyrA clpX*	$(1.27 \pm 0.24) \times 10^{-5}$	0.95 ± 0.21
*Eco*AI system[c]		
WA2899	$(1.25 \pm 0.11) \times 10^{-2}$	1.00 ± 0.25
WA2899 *clpP*	$(2.89 \pm 0.32) \times 10^{-3}$	0.87 ± 0.08
WA2899 *clpX*	$(1.84 \pm 0.17) \times 10^{-3}$	0.90 ± 0.10

a. The data represented are based on three independent experiments.
b. E.o.p. was calculated relative to NM840 Δ*hsdRM* derivative of C600 *gyrA*.
c. E.o.p. was calculated relative to NM863, $hsd_A R^- M^+ S^+$ derivative of WA2899.

affect the relative stabilities of the restriction enzyme ($R_2M_2S_1$) and the modification component (M_2S_1). If this were so, in the absence of ClpXP the loss of *hsd* genes, like the loss of type II R–M genes, could lead to 'programmed cell death'.

The fate of *clpP* and *clpX* cells after the loss of functional *hsd* genes by P1 transduction was assessed. Donor strains with deletions in *hsd* and a closely linked, selectable marker (*zjj*::Tn*10* or *dnaC*$^+$) were used. Tc^r, or DnaC$^+$, transductants were selected and scored for their restriction phenotype. If ClpXP was needed to prevent cell death, r^-m^- transductants of *clp*$^-$ recipients would be rare. A donor (NK231) with a deletion of *hsdM* and *S* was used for the *Eco*KI system and the frequency of r^-m^- transductants was unaffected by mutations in either *clpP* or *clpX*; the frequencies were 0.24, 0.22 and 0.24, respectively, for *clpP* (NK115), *clpX* (NK116) and *clp*$^+$ (NK31). In the experiment for the *Eco*AI system the donor (NM789) had a deletion in *hsdS*. The frequency of r^-m^- transductants was unaffected by either of the *clp* mutations; the frequencies of co-transduction were 0.26, 0.22 and 0.24 for *clpP* (NK219), *clpX* (NK220) and *clp*$^+$ (NM858) respectively. These data show that the *clp* mutations do not impede the loss of functional R–M genes for either *Eco*KI or *Eco*AI, despite the fact that the deletion in each donor strain left the *hsdR* gene functional. Even in the absence of ClpXP, the residual *Eco*KI and *Eco*AI do not lead to cell death when the coding sequences for the modification enzymes are lost.

Does the hsdC *derivative of* E. coli *C have a defect in* clpX *or* clpP*?*

Complementation tests were carried out using λ phages including wild-type and mutant *clp* genes. Four λ*clp* phages (*clpP*$^+$*X*$^+$, *clpP*$^+$*X*$^-$, *clpP*$^-$*X*$^+$ and *clpP*$^-$*X*$^-$) were used to lysogenize the $r^-m^-Clp^-$ derivatives of *E. coli* K-12 and the *hsdCrecA*$^+$ derivative of *E. coli* C. Integration was dependent on homologous recombination as the phages were *int*$^-$. The lysogens were used as recipients for conjugative transfer of the F′ plasmids that included the *hsd* genes from *E. coli* K–12. The expected complementation of known *clp* mutations (*clpP*$^-$*X*$^+$ + *clpP*$^+$*X*$^-$) was recognized by the efficient acquisition of F′ plasmids encoding functional *Eco*KI. Tests on lysogenic derivatives of the *hsdC* strain were consistent with a defect in *clpX* but not *clpP*; both λ*clpP*$^+$*X*$^+$ and λ*clpP*$^-$*X*$^+$, but neither λ*clpP*$^+$*X*$^-$ nor λ*clpP*$^-$*X*$^-$, complemented the *hsdC* lesion. These results agree with experiments carried out using multicopy plasmids as vectors for the *clp* genes (J. Ryu, personal communication).

Discussion

A sequential production of the modification and restriction enzymes is imperative when the genes encoding these activities are transferred to a recipient that lacks the appropriate protective modification of susceptible target sequences within its genome. For type II systems, in which the modification and restriction activities reside in different enzymes rather than both sharing a common component, there is evidence for the existence of regulatory genes, candidates for the transcriptional control of gene expression (Ives *et al.*, 1992; Tao and Blumenthal, 1992). There is no evidence for transcriptional control of expression of genes for type I and type III systems, regardless of whether they are encoded by plasmid, phage or chromosomal genes. Nevertheless, their transmission is efficient. One exception, where transmission was not detected, is the chromosomally encoded type III system, *Sty*LTI (de Backer and Colson, 1991). Consistent with resistance to transmission, and presumably therefore to change of specificity, no allelic diversity has been detected for the locus including the genes specifying *Sty*LTI. This contrasts with the *hsd* genes encoding the type IA system *Sty*LTIII; these *hsd* genes are alleles of those specifying *Eco*KI and *Eco*AI.

The role of proteases as an alternative mechanism for the control of gene expression is currently becoming well documented in both prokaryotes and eukaryotes. Early lines of evidence for this type of post-transcriptional control were provided from studies of the developmental pathways of phages (see, for example, Gottesman *et al.*, 1981). Recent biochemical experiments have shown that the CII regulatory protein of λ is degraded by the essential host protease FtsH, alias HflB (Kihara *et al.*, 1997; Shotland *et al.*, 1997), and that the Mu repressor is a target for the ClpXP protease (Laachouch *et al.*, 1996). The complex,

hetero-oligomeric type I and type III R–M systems offer specific targets that would enable proteases to elicit temporal control of the two activities. The simplest model would require that one subunit of a complex restriction endonuclease, a subunit essential for restriction but not modification, is the target for proteolysis. The association of this subunit with the component essential for modification – the Mod subunit of a type III enzyme or M_2S_1 complex of a type I system – would protect the sensitive subunit – Res for a type III system and HsdR or R for a type I – from proteolysis.

Redaschi and Bickle (1996) used Western blots to monitor the production of the Res subunit of the type III systems *Eco*P1I and *Eco*P15I. They detected very little Res in the absence of Mod and suggested that the Mod subunit may regulate the amount of Res by protecting it from degradation by proteases. Some mechanism of control for type I systems was anticipated from early observations that the genes encoding the modification component of a type IB system, *Eco*AI, could not be transferred to a recipient containing a plasmid expressing the *hsdR* gene (Fuller-Pace *et al.*, 1985; Suri and Bickle, 1985). More recently, it has been demonstrated that the efficient establishment of the genes encoding a type IA system (Prakash-Cheng *et al.*, 1993) or a type IB system (Kulik and Bickle, 1996) in a new host is dependent on the product of the *hsdC* gene of *E. coli*, whereas the plasmid-encoded genes for a member of the IC family are efficiently transferred even to an *hsdC*$^-$ recipient (Kulik and Bickle, 1996). Our experiments identify both polypeptides of the ClpXP protease as necessary for the efficient acquisition of the genes encoding either *Eco*KI or *Eco*AI. A simple explanation of this result is that the ClpXP protease, or a component of this complex, competes with the methylase (M_2S_1) for interaction with HsdR, thereby delaying the production of the complex with endonuclease activity.

The *clpX* and *P* alleles used in our experiments are well-characterized null mutations. Our data show that ClpX and ClpP are both relevant to the transmission of *hsd* genes for members of both the type IA and IB families of R–M systems but that the *clpX* mutation imposes a stronger barrier than *clpP*, implicating a dual role for ClpX, possibly as a chaperone and as a component of the ClpXP protease. Although efficient transmission of the hsd_K and hsd_A genes to other strains requires ClpX and ClpP, the *hsd* genes for *Eco*KI are more dependent than those for *Eco*AI. Furthermore, the strength of the barrier imposed by *clp* mutations is influenced by the mode of transmission: conjugation and transformation being more sensitive than P1 transduction. The relevance of the mode of gene transfer and the difference between families is accentuated by the P1 transduction experiments. In the case of *Eco*AI, no barrier was detectable even for a *clpX* recipient (Table 5). Our data, particularly for transformation (see Table 3) indicate a difference between the phenotype of the *clpX* mutation in *E. coli* K-12 and the phenotype of the *hsdC* mutation in the *recA*$^+$ derivative of JR302 (NM820). The difference may reflect an additional mutation or the different genetic backgrounds. Genetic analysis of JR302 is hindered by the fact that JR302 is a hybrid of *E. coli* C and *E. coli* K-12.

The hsd_A genes may be more amenable to transfer because the modification component of this system is active on unmethylated target sequences (Suri and Bickle, 1985), whereas that of *Eco*KI has a very strong preference for hemimethylated DNA (Dryden *et al.*, 1993). The relevance of the mode of gene transfer is not known, but it is possible that cells receiving DNA from P1 capsids are less sensitive to restriction, less vulnerable to DNA degradation, or both. It is known that the Dar function of P1 protects the donor DNA from restriction enzymes present in the recipient cell (Iida *et al.*, 1987). The same, or another, function could provide some protection against a restriction activity encoded by donor genes.

The current thinking about ATP-dependent proteases, including ClpXP, is one in which a structural component of the protease has intrinsic chaperone activity and it has been suggested that the initial steps in energy-dependent protein degradation may be similar to those of chaperone-dependent protein folding. Gottesman *et al.* (1997) have proposed a general model for handling misfolded proteins *in vivo* in which either swift refolding of proteins with functional potential is achieved or irreversibly denatured and damaged proteins are rapidly degraded. If this model is applied to type I R–M systems, on the assumption that HsdR subunits are the substrate for the ClpXP protease, a failure to stabilize an inactive polypeptide would lead to loss of restriction potential. In fact, our experiments show that restriction is enhanced in a *clpX* bacterium, consistent with increased levels of functional *Eco*KI ($R_2M_2S_1$). This result implies that ClpX chaperone activity is not essential for endonuclease activity, rather that ClpX may play the opposite role of inducing a change from the functional form of HsdR to one that becomes a substrate for degradation by the ClpXP protease. This role could be one in which HsdR is destabilized, making it a substrate for protease activity, or one in which HsdR is either prevented from interaction with or made unsuitable for interaction with the modification methylase. Association of HsdR with the methylase could block access of ClpX to its target sequence. Precedence for explanations of this sort have been provided recently for the MuA transposase in which target sequences for ClpX and MuA overlap (Levchenko *et al.*, 1997). Although potential target sequences in the HsdR polypeptide can be identified, it is premature to speculate. It remains to be determined whether HsdR itself is a substrate for the ClpXP protease, but the identification of ClpXP as a host factor facilitating the transmission of type

Table 7. Bacterial strains.

Strain	Relevant genotype or phenotype	Source or origin
E. coli K-12		
AB1157	$r_K^+m_K^+$ *rac*-0	De Witt and Adelberg (1962)
C600	$r_K^+m_K^+$	Appleyard (1954)
5K	C600 *hsdR514*	Hubacek and Glover (1970)
JC9935	AB1157 *recA13 sup*^{c}	A. J. Clark
KL719	F′ 101 ($hsd_KR^+M^+S^+$)	Low (1968)
NM477	C600 *ΔhsdMS*	Gough and Murray (1983)
NM654	C600 *ΔhsdRM*	Loenen *et al.* (1987)
NM789	$\Delta hsdS_A$	Thorpe *et al.* (1997)
TPC48	*dnaC325 zjj*::Tn*10*	Masters *et al.* (1989)
WA2552	*hsdR* ($r_A^-m_A^+$)	Arber and Wauters-Willems(1970)
WA2899	C600 $r_K^-m_K^-r_A^+m_A^+$	Fuller-Pace *et al.* (1985)
XL1-Blue	*recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* ($r_K^-m_K^+$) *supE44 relA1 lacF′* [Tn*10* (Tc^r) *proAB lacI*^{q}*ΔlacZM15*]	Bullock *et al.* (1987)
SG20252	MC4100 *lon-100 zba-3000*::Tn*10*	Trisler and Gottesman (1984)
SG21173	MC4100 *ΔclpA::kan*	Gottesman (1990)
SG22007	MC4100 *ΔclpP1*::*cat*	Maurizi *et al.* (1990)
SG22080	MC4100 *ΔclpX1*::*kan*	Gottesman *et al.* (1993)
SG22129	MC4100 *ΔclpP1*::*cat ΔclpX1*::*kan*	S. Gottesman
SG22192	MC4100 *ΔclpQ*::*cat*	W.-F.Wu and S.Gottesman
SG22193	MC4100 *ΔclpY*::*cat*	W.-F.Wu and S.Gottesman
MA156	*hflA150* Tn*10**	M. A. Hoyt
WA8304	*hflA150 hflB29 zgj25*::Tn*10*	Banuett *et al.* (1986)
NK31	C600 *gyrA96*	C600×P1(XL1-Blue)
NM840	C600 *gyrA96 ΔhsdRM* ($r_K^-m_K^-$)	NK31×P1(NM654)
NM858	WA2899 *dnaC325 zjj*::Tn*10*	WA2899×P1(TPC48)
NM863	WA2899 *hsdR*	NM858×P1(WA2552)
NK121	NM840 *ΔclpP1*::*cat*	NM840×P1(SG22007)
NK123	NM840 *ΔclpX1*::*kan*	NM840×P1(SG22080)
NK152	NM840 *lon-100 zba-3000*::Tn*10*	NM840×P1(SG20252)
NK188	NM840 *ΔclpA*::*kan*	NM840×P1(SG21173)
NK190	NM840 *ΔclpQ*::*cat*	NM840×P1(SG22192)
NK191	NM840 *ΔclpY*::*cat*	NM840×P1(SG22193)
NK228	NM840::Tn*10 hflA150**	NM840×P1(MA156)
NK229	NM840 *hflB29 zgj25*::Tn*10*	NM840×P1(WA8304)
NK113	AB1157 *ΔclpP1*::*cat*	AB1157×P1(SG22007)
NK114	AB1157 *ΔclpX1*::*kan*	AB1157×P1(SG22080)
NK115	C600 *gyrA96 ΔclpP1*::*cat*	NK31×P1(SG22007)
NK116	C600 *gyrA96 ΔclpX1*::*kan*	NK31×P1(SG22080)
NK125	NM840 *dnaC325 zjj*::Tn*10*	NM840×P1(TPC48)
NK122	NK125 *ΔclpP1*::*cat*	NK125×P1(SG22007)
NK124	NK125 *ΔclpX1*::*kan*	NK125×P1(SG22080)
NK167	NM840 $hsd_AR^+M^+S^+$ *zjj*::Tn*10*	NK125×P1(WA2899)
NK170	NM840 $hsd_AR^-M^+S^+$ *zjj*::Tn*10*	NK125×P1(NM863)
NK219	NM858 *ΔclpP1*::*cat*	NM858×P1(SG22007)
NK220	NM858 *ΔclpX1*::*kan*	NM858×P1(SG22080)
NK231	NM477 *gyrA96 zjj*::Tn*10*	NM477 *gyrA*×P1(TPC48)
NK233	WA2899 *ΔclpP1*::*cat*	WA2899×P1(SG22007)
NK234	WA2899 *ΔclpX1*::*kan*	WA2899×P1(SG22080)
E. coli C		
JR300	wild type r^{c}m^c	Prakash-Cheng *et al.* (1993)
JR302	JR300 *recA hsdC* Km^r	Prakash-Cheng *et al.* (1993)
NK38	JR300 *gyrA96*	JR300×P1(XL1-Blue)
NM820	JR302 *recA*$^+$	O'Neill *et al.* (1997)
NM822	NM820 $hsd_KR^-M^+S^+$ *dnaC325 zjj*::Tn*10 hsdC*	O'Neill *et al.* (1997)
NM824	JR300 $hsd_KR^-M^+S^+$ *dnaC325 zjj*::Tn*10*	O'Neill *et al.* (1997)

* Where Tn*10* is linked to *hflA150*.

I R–M genes enables investigation of the role of ClpX and ClpP in the sequential control of the modification and restriction activities at a molecular level.

Experimental procedures

Bacterial strains

These are listed in Table 7.

Plasmids

F′101-201 and F′101-202, respectively, are hsd_K^+ and $hsdR^-$ Cm^r derivatives of F′101-101 and F′101-102 (Prakash-Cheng *et al.*, 1993). Chloramphenicol resistance, conferred by miniTn*5*-Cm, was transposed from the mobilizable vector pUT/Cm as described by de Lorenzo *et al.* (1990).

F′ plasmids with hsd_A^+ genes (F′101-301) and an $hsdR^-$ derivative (F′101-302) were isolated following the conjugative transfer of F′101 (Low, 1972) to NK167 ($r_A^+m_A^+$) and NK170 ($r_A^-m_A^+$) respectively, recipient strains with a Tc^r marker (*zjj*::Tn*10*) linked to the chromosomal hsd_A genes. F′101 includes a chromosomal segment of the *E. coli* K-12 genome that spans *hsd* and *zjj*::Tn*10*. Recombinant derivatives in which the hsd_K genes on the F′ had been replaced by the hsd_A genes were detected by transfer of the F′ plasmids to the *recA* strain JC9935. Tc^r Sm^r conjugants were tested for restriction and modification by the *Eco*AI system and for their ability to co-transfer *tet* and the hsd_A genes. MiniTn*5*-Cm derivatives of the new plasmids were selected as described above.

Other plasmids were derivatives of pBR322. One pair ($phsd^+$ and $phsdR^-$) includes the *hsd* genes of *E. coli* K-12 differing only by a missense mutation in *hsdR* (O'Neill *et al.*, 1997). Similarly, pFFP30 includes the three functional *hsd* genes encoding *Eco*AI, and pFFP31 lacks the *hsdR* gene (Fuller-Pace *et al.*, 1985). pBRK has a *kan* gene inserted at the *Pst*I site in the *bla* gene, thereby conferring an Ap^sKm^r phenotype. pWPC16 includes $clpX^+$ and *clpP*::*cat* (Maurizi *et al.*, 1990).

Phages

λ*vir* was used as a test phage for restriction and modification systems. Other phages were the following *clp* derivatives of the λ vector NM1151 (Murray, 1983). The $\lambda clpP^+X^+$ (λNM1357) includes a 6.2 kb *Bam*HI fragment from the Kohara phage 148 (Kohara *et al.*, 1987), a *clpX*::*kan* derivative (λNM1361) has the homologous *Bam*HI fragment from NM840*clpX*, and a *clpP*::*cat* derivative (λNM1359) was made by inserting the *Hin*dIII–*Bam*HI fragment from pWPC16 (Maurizi *et al.*, 1990); this $\lambda clpP^-X^+$ phage, like pWPC16, has a deletion that extends from within the *tig* gene, upstream of *clpP* into *bolA*, leaving *clpX* as the only functional gene within the cloned DNA fragment. λ*clpP*::*cat clpX*::*kan* (λNM1362) was made *in vivo* by excision of the prophage from the *clpP*::*cat clpX*::*kan* double mutant (SG22129) lysogenic for λ*clpP*::*cat* (λNM1359).

Microbial methods

Media and general methods have been described (Murray *et al.*, 1977). P1 transductions were carried out according to Miller (1992) and conjugation experiments followed the procedures described by Prakash-Cheng *et al.* (1993). Cells were made competent in the uptake of DNA by electroporation using a Gene Pulser (Bio-Rad). Some lysogens could be selected as Cm^r or Km^r colonies; if not they were selected as immune colonies at 32°C, using $\lambda b2\ imm^{21}cI^-$ and h^{82} *b*522 imm^{21} cI^- phages for selection.

Acknowledgements

We thank Mary O'Neill and David Dryden for helpful comments and Natalie Honeyman for her patient preparation of the manuscript. We are indebted to Susan Gottesman, Ariane Toussaint and David Leach for providing strains and the Medical Research Council for continued support. Svetlana Makovets thanks the Darwin Trust for the award of a scholarship.

References

Appleyard, R.K. (1954) Segregation of lambda lysogenicity during bacterial recombination in *Escherichia coli* K-12. *Genetics* **39:** 440–452.

Arber, W., and Wauters-Willems, D. (1970) Host specificity of DNA produced by *Escherichia coli*. XII. The two restriction and modification systems of strain 15T. *Mol Gen Genet* **108:** 203–217.

Banuett, F., Hoyt, M.A., McFarlane, L., Echols, H., and Herskovitz, I. (1986) *hflB*, a new *Escherichia coli* locus regulating lysogeny and the level of bacteriophage lambda cII protein. *J Mol Biol* **187:** 213–224.

Barcus, V.A., and Murray, N.E. (1995) Barriers to recombination: restriction. In *Population Genetics of Bacteria*. Cambridge, UK: Cambridge University Press, pp. 31–58.

Barcus, V.A., Titheradge, A.J.B., and Murray, N.E. (1995) The diversity of alleles at the *hsd* locus in natural populations of *Escherichia coli*. *Genetics* **140:** 1187–1197.

Bullock, W.O., Fernandez, J.M., and Short, J.M. (1987) XLI-Blue: A high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. *BioTechniques* **5:** 376–379.

De Backer, D., and Colson, C. (1991) Transfer of genes for the *Sty*LTI restriction-modification system of *Salmonella typhimurium* to strains lacking modification ability results in death of the recipient cells and degradation of their DNA. *J Bacteriol* **173:** 1328–1330.

De Lorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K.N. (1990) Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol* **172:** 6568–6572.

De Witt, S.K., and Adelberg, E.A. (1962) The occurrence of a genetic transposition in a strain of *Escherichia coli*. *Genetics* **47:** 577.

Dryden, D.T.F., Cooper, L.P., and Murray, N.E. (1993) Purification and characterisation of the methyltransferase from the type I restriction and modification system of *Escherichia coli* K12. *J Biol Chem* **268:** 13228–13236.

Dryden, D.T.F., Cooper, L.P., Thorpe, P.H., and Byron, O. (1997) The *in vitro* assembly of the *Eco*KI type I DNA restriction/modification enzyme and its *in vivo* implications. *Biochemistry* **5:** 1065–1076.

Fuller-Pace, F.V., Cowan, J.M., and Murray, N.E. (1985) *Eco*A and *Eco*E: alternatives to the *Eco*K family of type I restriction and modification systems of *Escherichia coli*. *J Mol Biol* **186:** 65–75.

Gottesman, S. (1990) Minimizing proteolysis in *Escherichia coli*: genetic solutions. *Methods Enzymol* **185:** 119–129.

Gottesman, S. (1996) Proteases and their targets in *Escherichia coli*. *Annu Rev Genet* **30:** 465–506.

Gottesman, S., Gottesman, M.E., Shaw, J.E., and Pearson, M.L. (1981) Protein degradation in *E. coli*: the *lon* mutation and bacteriophage lambda N and CII protein stability. *Cell* **24:** 225–233.

Gottesman, S., Clark, W.P., de Crecy-Lagard, V., and Maurizi, M.R. (1993) ClpX, an alternative subunit for the ATP-dependent Clp protease of *Escherichia coli*. *J Biol Chem* **268:** 22618–22626.

Gottesman, S., Wickner, S., and Maurizi, M.R. (1997) Protein quality control: triage by chaperones and proteases. *Genes Dev* **11:** 815–823.

Gough, J.A., and Murray, N.E. (1983) Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J Mol Biol* **166:** 1–19.

Hobbs, M., and Reeves, P.R. (1994) The JUMPstart sequence: a 39 bp element common to several polysaccharide gene clusters. *Mol Microbiol* **12:** 855–856.

Hubacek, J., and Glover, S.W. (1970) Complementation analysis of temperature-sensitive host specificity mutations in *Escherichia coli*. *J Mol Biol* **50:** 111–127.

Iida, S., Streigg, M.B., Bickle, T.A., and Arber, W. (1987) Two DNA antirestriction systems of bacteriophage P1, *darA*, and *darB*: characterization of *darA*$^-$ phages. *Virology* **157:** 156–166.

Ives, C.L., Nathan, P.D., and Brooks, J.E. (1992) Regulation of the *Bam*HI restriction modification system by a small intergenic ORF, *bamHIC*, in both *Escherichia coli* and *Bacillus subtilis*. *J Bacteriol* **174:** 7194–7201.

Kihara, A., Akiyama, Y., and Ito, K. (1997) Host regulation of lysogenic decision in bacteriophage λ: transmembrane modulation of FtsH (HflB), the cII degrading protease, by HflKC (HflA). *Proc Natl Acad Sci USA* **94:** 5544–5549.

Kohara, Y., Akiyama, K., and Isono, K. (1987) The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50:** 495–508.

Kulakauskas, S., Lubys, A., and Ehrlich, S.D. (1995) DNA restriction-modification systems mediate plasmid maintenance. *J Bacteriol* **177:** 3451–3454.

Kulik, E.M., and Bickle, T.A. (1996) Regulation of the activity of the type IC *Eco*R124I restriction enzyme. *J Mol Biol* **264:** 891–906.

Laachouch, J.E., Desmet, L., Geuskens, V., Grimaud, R., and Toussaint, A. (1996) Bacteriophage Mu repressor as a target for the *Escherichia coli* ATP-dépendent Clp protease. *EMBO J* **15:** 437–444.

Levchenko, I., Luo, L., and Baker, T.A. (1995) Disassembly of the Mu transposase tetramer by the ClpX chaperone. *Genes Dev* **9:** 2399–2408.

Levchenko, I., Yamauchi, M., and Baker, T.A. (1997) ClpX and MuB interact with overlapping regions of Mu transposase: implications for control of the transposition pathway. *Genes Dev* **11:** 1561–1572.

Loenen, W.A.M., Daniel, A.S., Braymer, H.D., and Murray, N.E. (1987) Organization and sequence of the *hsd* genes of *Escherichia coli K-12*. *J Mol Biol* **198:** 159–170.

Low, K.B. (1968) Formation of merodiploids in matings with a class of Rec$^-$ recipient strains of *Escherichia coli* K12. *Proc Natl Acad Sci USA* **60:** 160–167.

Low, K.B. (1972) *Escherichia coli* K-12 F-prime factors, old and new. *Bacteriol Rev* **36:** 587–607.

Masters, M., Paterson, T., Popplewell, A.G., Owen-Hughes, T., Pringle, J.H., and Begg, K.J. (1989) The effect of DnaA protein levels and the rate of initiation at *oriC* on transcription originating in the *ftsQ* and *ftsA* genes: *in vivo* experiments. *Mol Gen Genet* **216:** 475–483.

Maurizi, M.R., Clark, W.P., Katayama, Y., Rudikoff, S., Pumphrey, J., Bowers, B., and Gottesman, S. (1990) Sequence and structure of ClpP, the proteolytic component of the ATP-dependent Clp protease of *Escherichia coli*. *J Biol Chem* **265:** 12536–12545.

Milkman, R. (1997) Recombination and population structure in *Escherichia coli*. *Genetics* **146:** 745–750.

Miller, J.H. (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Murray, N.E. (1983) Phage lambda and molecular cloning. In *Lambda II*. Hendrix, R.W., Roberts, J.W., Stahl, F.W., and Weisberg, R.A. (eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 395–432.

Murray, N.E., Brammar, W.J., and Murray, K. (1977) Lambdoid phages that simplify the recovery of *in vitro* recombinants. *Mol Gen Genet* **150:** 53–61.

Naito, T., Kusano, K., and Kobayashi, I. (1995) Selfish behaviour of restriction-modification systems. *Science* **267:** 897–899.

O'Neill, M., Chen, A., and Murray, N.E. (1997) The restriction-modification genes of *E. coli* K-12 may not be selfish; they do not resist loss and are readily replaced by alleles conferring different specificities. *Proc Natl Acad Sci USA* **94:** 14596–14601.

Prakash-Cheng, A., and Ryu, J. (1993) Delayed expression of *in vivo* restriction activity following conjugal transfer of *Escherichia coli* hsd_K genes. *J Bacteriol* **175:** 4905–4906.

Prakash-Cheng, A., Chung, S.S., and Ryu, J.-I. (1993) The expression and regulation of *hsdK* genes after conjugative transfer. *Mol Gen Genet* **241:** 491–496.

Redaschi, N., and Bickle, T.A. (1996) Posttranscriptional regulation of *Eco*P1I and *Eco*P15I restriction activity. *J Mol Biol* **257:** 790–803.

Shotland, Y., Koby, S., Teff, D., Mansur, N., Oren, D.A., Tatematsu, K., *et al.* (1997) Proteolysis of the phage (CII regulatory protein by FtsH (HflB) of *Escherichia coli*. *Mol Microbiol* **24:** 1303–1310.

Suri, B., and Bickle, T.A. (1985) *Eco*A: the first member of a new family of type I restriction modification systems. Gene organization and enzymatic activities. *J Mol Biol* **186:** 77–85.

Tao, T., and Blumenthal, R.M. (1992) Sequence and

characterization of *pvuIIR*, the *Pvu*II endonuclease gene, and of *pvuIIC*, its regulatory gene. *J Bacteriol* **174:** 3395–3398.

Thorpe, P.H., Ternent, D., and Murray, N.E. (1997) The specificity of *Sty*SKI, a type I restriction enzyme, implies a structure with rotational symmetry. *Nucleic Acids Res* **25:** 1694–1700.

Trisler, P., and Gottesman, S. (1984) *lon* transcriptional regulation of genes necessary for capsular polysaccharide synthesis in *Escherichia coli* K-12. *J Bacteriol* **160:** 184–191.

Webb, J.L., King, G., Ternent, D., Titheradge, A.J.B., and Murray, N.E. (1996) Restriction by *Eco*KI is enhanced by co-operative interactions between target sequences and is dependent on DEAD box motifs. *EMBO J* **15:** 2003–2009.

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 9757–9762, August 1999
Genetics

Regulation of endonuclease activity by proteolysis prevents breakage of unmodified bacterial chromosomes by type I restriction enzymes

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Communicated by Allan Campbell, Stanford University, Stanford CA, June 3, 1999 (received for review April 19, 1999)

ABSTRACT **ClpXP-dependent proteolysis has been implicated in the delayed detection of restriction activity after the acquisition of the genes (*hsdR*, *hsdM*, and *hsdS*) that specify *Eco*KI and *Eco*AI, representatives of two families of type I restriction and modification (R-M) systems. Modification, once established, has been assumed to provide adequate protection against a resident restriction system. However, unmodified targets may be generated in the DNA of an *hsd*$^+$ bacterium as the result of replication errors or recombination-dependent repair. We show that ClpXP-dependent regulation of the endonuclease activity enables bacteria that acquire unmodified chromosomal target sequences to survive. In such bacteria, HsdR, the polypeptide of the R-M complex essential for restriction but not modification, is degraded in the presence of ClpXP. A mutation that blocks only the modification activity of *Eco*KI, leaving the cell with ≈600 unmodified targets, is not lethal provided that ClpXP is present. Our data support a model in which the HsdR component of a type I restriction endonuclease becomes a substrate for proteolysis after the endonuclease has bound to unmodified target sequences, but before completion of the pathway that would result in DNA breakage.**

Within a bacterium that has a classical restriction and modification (R-M) system, the nucleotide sequences that define the targets for attack by the resident restriction endonuclease are concealed by the modification of appropriate bases within them. For some systems this modification is achieved by the methylation of specific adenine residues, and for others it is achieved by methylation of cytosine residues. The restriction endonuclease has the potential to attack DNA from different strains of the same species because foreign DNA generally lacks the protective imprint of the relevant methyltransferase (for reviews see refs. 1 and 2). Restriction of the host cell's newly synthesized DNA normally is avoided, because the unmethylated strand of each target sequence produced by DNA replication is methylated before the next round of replication. If, however, resident DNA were to acquire unmodified target sequences, would it, like foreign DNA, become a substrate for restriction? In this paper we show that in situations where the modification of the host DNA by a type I R-M system fails, an alternative level of protection impairs the endonuclease activity of the restriction system and the bacteria survive.

A type I R-M system is encoded by three genes: *hsdR*, *hsdM*, and *hsdS*. The three polypeptides, HsdR, HsdM, and HsdS, often designated R, M, and S, assemble to give an enzyme ($R_2M_2S_1$) that modifies hemimethylated DNA and restricts unmethylated DNA. A smaller complex (M_2S_1) has only the methyltransferase activity. The S subunit confers target specificity; hence, both complexes and both activities respond to the same nucleotide sequence.

Type I systems of enteric bacteria have been divided into discrete families by tests for cross-hybridization between genes and cross-reactivity with antibodies raised against the archetypal member of each family (3–5). Four families of distantly related systems have been identified (types IA, IB, IC, and ID), and where complementation tests have been done they indicate that enzymes in the same family can interchange subunits, but those from different families cannot (6, 7).

No transcriptional regulation of type I R-M genes has been detected; yet these genes are transferred readily to recipient bacteria devoid of the relevant modification activity (8–10). It is presumed that the cells survive the acquisition of the new R-M system because they become restriction proficient only after the modification activity is established. Experiments in support of this identify a lag of ≈15 generations before the cells become restriction-proficient after the acquisition of *hsd* genes by conjugation (11). The ClpXP protease was shown to be essential for the effective acquisition of genes specifying type IA and IB systems, and for this reason proteolysis has been implicated in the delayed expression of restriction activity (10).

The acquisition of a new specificity system is not the only situation in which a temporary loss of restriction proficiency has been detected. A well documented example, referred to as restriction alleviation (RA), occurs in response to treatments that damage DNA (12–14). UV light, nalidixic acid, and 2-aminopurine (2-AP) have been shown to induce restriction alleviation. It is possible that the temporary loss of restriction proficiency associated with the establishment of a new specificity is an example of RA. If this is so, ClpXP would be required for the alleviation of restriction in response to DNA damage. We have tested this hypothesis and show ClpXP to be a common requirement for RA in response to the various agents that damage DNA. This led us to identify steps in the molecular pathway that protect bacteria against the potentially lethal effects of restriction after DNA damage in a cell with a resident type I system or after the acquisition of a type I system capable of attacking the resident DNA.

MATERIALS AND METHODS

Bacterial Strains, Phages, Plasmids, and General Microbial Methods. Bacterial strains are listed in Table 1. Integration-deficient, λ*hsdc*I857 phages were used to transfer *hsd* alleles to bacterial chromosomes: λNM1367 includes *hsd*Δ*RM*(F269G)*S*$^+$; λNM1376, *hsdM*$^+$*S*$^+$; λNM1394, *hsdM*(F269G)*S*$^+$; and λNM1384, *hsdR*(A619V) (17). JC9935 was used as the donor of the following derivatives of F′101: F′101–102, $hsd_KR^-M^+S^+$ (11); F′101–301, hsd_K^- hsd_A^+ (10);

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Abbreviations: R-M, restriction and modification; RA, restriction alleviation; 2-AP, 2-aminopurine; DSB, double-strand break; EOP, efficiency of plating.

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Table 1. *E. coli* K-12 strains

Strain	Relevant genotype	Source or origin
C600	hsd_K^+	See ref. 10
5K	*hsdR514*	See ref. 10
CB51	*dam-3*	C. Boyd
JC9935	*recA13*	See ref. 10
LE451	*rac-0 recA srl*::Tn*10*	Ref. 15
NM477	Δ(*hsdMS*)*5*	See ref. 10
NM659	Δ*recA*::*cat*	This laboratory
NM679	Δ(*hsdRMS*)	Ref. 16
NM799	*hsdR*(A619V)	Refs. 17 and 18
NM802	Δ*hsdR4*	This laboratory
SG22007	Δ*clpP*::*cat*	Ref. 19
SG22080	Δ*clpX*::*kan*	Ref. 20
SG22129	Δ*clpP*::*cat* Δ *clpX*::*kan*	S. Gottesman
RH6972	*dnaQ*::*mini*Tn*10* (*mutD*)	D. R. F. Leach
RS2	*topA10*	Ref. 21
TPC48	*zjj*::Tn*10 dnaC*^{ts}	See ref. 10
NK31	*gyrA96*	Ref. 10
NK167	$hsd_K^-hsd_A^+$	Ref. 10
NK300	*rac-0 recA*$^+$*srl*$^+$	LB451 × P1(C600)
NK301	*rac-0 gyrA96*	NK300 × P1(NK31)
NK302	*dam*	NK301 × P1(CB51)
NK303	Δ*clpP*	NK301 × P1(SG22007)
NK304	Δ*clpX*	NK301 × P1(SG22080)
NK308	Δ*recA*	NK301 × P1(NM659)
NK309	*zjj*::Tn10 *dnaC*^{ts}	NK301 × P1(TPC48)
NK310	*hsdR*	NK301 × P1(5K)
NK311	Δ(*hsdRMS*)	NK309 × P1(NM679)
NK312	Δ(*hsdRMS*) Δ*clpX*	NK311 × P1(SG22080)
NK315	*dam* Δ*clpX*	NK302 × P1(SG22080)
NK320	Δ*clpX*	NK300 × P1(SG22080)
NK323	Δ*clpX* Δ*recA*	NK304 × P1(NM659)
NK324	Δ(*hsdRMS*) Δ*clpX* Δ*recA*	NK312 × P1(NM659)
NK325	*hsdR* Δ*clpX*	NK310 × P1(SG22080)
NK326	*mutD*	NK301 × P1(RH6972)
NK327	*mutD* Δ*clpX*	NK326 × P1(SG22080)
NK329	*topA10* Δ*clpP* Δ*clpX*	RS2 × P1(SG22129)
NK351	*hsdR*(A619V)	NK309 × P1(NM799)
NK352	Δ(*hsdMS*)*5*	NK309 × P1(NM477)
NK354	$hsd_K^-hsd_A^+$	NK309 × P1(NK167)
NK355	$hsd_K^-hsd_A^+$ Δ*clpX*	NK354 × P1(SG22080)
NK378	Δ*hsdR hsdM*(F269G)	NM802 × λNM1367
NK379	Δ*hsdR*	NK309 × P1(NM802)
NK380	Δ*hsdR* Δ*clpX*	NK379 × P1(SG22080)
NK382	Δ*hsdR hsdM*(F269G)	NK309 × P1(NK378)
NK383	Δ*hsdR hsdM*(F269G) Δ*recA*	NK382 × P1(NM659)
NK384	Δ*hsdR hsdM*(F269G) Δ*clpX*	NK382 × P1(SG22080)
NK386	*hsdM*(F269G)	NK301 × λNM1394
NK388	*hsdR*(A619V)*hsdM*(F269G)	NK386 × λNM1384

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and F′101–103, *zjj*::Tn*10* hsd_KR^+Δ*(MS)5*. F′101–103 was selected after plasmid–chromosome allele exchange, as described for F′101–301 (10). pNK3 was made by transferring the *Hin*dIII-*Sma*I fragment containing *hsdR* from pBg3 (22) to pACYC184 (23) digested with *Hin*dIII and *Nru*I. Media and general methods were as described previously (10).

Restriction Alleviation. 2-AP (400 μg/ml) was added to midlogarithmic cultures grown at 37°C in LB medium. Intensive aeration was provided before and during the treatment. After 1 h, the cells were washed, resuspended in fresh broth, and tested for restriction. UV-induced RA was measured as described in ref. 24, and RA in response to nalidixic acid was measured as described by (13).

Analysis of Proteins. Polypeptides were separated by electrophoresis through SDS/polyacrylamide gels (25). Western blots used rabbit antisera against *Eco*KI or *Eco*AI and the chemiluminescence detection system (POD) of Boehringer Mannheim.

The stability of proteins was monitored after pulse-labeling with [^{35}S]methionine. Bacteria were grown at 37°C with intensive aeration to an OD_{600} of 0.2–0.3 in minimal medium supplemented with thiamin and all amino acids except methionine and cysteine. Chloramphenicol (20 μg/ml) maintained the presence of pNK3. Each culture was divided, and 2-AP (400 μg/ml) was added to one aliquot. After 1.5 h, a 1-min pulse of [^{35}S]methionine (25 μCi/ml) was given. Labeling was stopped by diluting each culture with an equal volume of prewarmed LB supplemented with L-methionine (15 μM) or with L-methionine and 2-AP (400 μg/ml). Intensive aeration was maintained, and samples were taken at appropriate intervals. Bacteria were collected by centrifugation, resuspended in SDS sample buffer, and boiled for 5 min, and samples were applied to SDS/polyacrylamide gels for the separation of polypeptides by electrophoresis.

RESULTS

ClpXP Is Necessary for RA. A simple quantitative test for restriction relies on the fact that most unmodified λ phages are killed when they infect *Escherichia coli* K-12; the phage genome is a substrate for *Eco*KI, the resident restriction system. The titer of an unmodified phage lysate (λ.0) on a restricting host relative to that on a nonrestricting derivative is referred to as the efficiency of plating (EOP). Therefore, the inverse of EOP quantifies restriction. RA is detected as a temporary reduction in restriction (hence, an increased EOP) after treatment of genetically restriction-proficient cells with agents that damage DNA.

We examined RA for Clp^+ and Clp^- strains in response to each of three treatments; UV light, nalidixic acid, and 2-AP. For each treatment, ClpX was essential for efficient RA (Fig. 1). A *clpP* strain was tested for RA in response to 2-AP, and it also was deficient in RA (data not shown). The results support our hypothesis that RA, in response to agents that damage DNA, and the delayed expression of restriction activity after the acquisition of hsd^+ genes by an hsd^- recipient are both the outcome of a common ClpXP-dependent process. RA for the *Eco*AI system in response to 2-AP also was shown to be dependent on ClpX (data not shown).

"Constitutive" RA. Restriction is alleviated in *dam* strains (26). It is known that the Dam-methylase identifies the parental DNA strand during mismatch repair, and in *dam* mutants mismatch repair leads to double-strand breaks (DSBs) (27). This alleviation of restriction in *dam* strains led us to question whether other mutations that impair the efficiency or fidelity of DNA replication might induce RA. If such a phenotype occurred, would it be dependent on ClpXP? We tested *topA, mutD*, and *dam* strains.

Mutants deficient in topoisomerase I, like wild-type cells treated with nalidixic acid, have problems in DNA replication. DSBs may occur when the replication forks stall (28). In

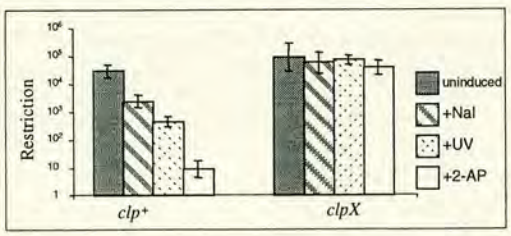

FIG. 1. Restriction of unmodified phage λ by *clp*$^+$ (NK301, NK300 for nalidixic acid) and *clpX* (NK304, NK320 for nalidixic acid) bacteria. Only *clp*$^+$ cells show restriction alleviation.

contrast, a *mutD* mutation enhances the error rate of DNA polymerase III (29) and the increased frequency of mismatches may mimic the effect of 2-AP, an analogue of adenine that causes base pair transitions.

Restriction by *dam*, *topA*, or *mutD* strains was at least 100-fold less efficient than restriction by wild-type *E. coli* K-12 (Fig. 2). If this poor restriction is the result of constitutive expression of RA activated in response to either DNA damage or mismatches, then a mutation in *clpX* or *clpP* should restore restriction. Consistent with this prediction, the efficiency of restriction was enhanced by approximately 100-fold in the absence of ClpXP protease (Fig. 2).

ClpXP-Deficient, Restriction-Proficient Bacteria Die During Prolonged Exposure to 2-AP. After prolonged treatment with 2-AP (3–4 h at 400 μg/ml), *clp*$^-$ (NK303 and NK304) but not *clp*$^+$ (NK301) bacteria become filamentous, a phenotype characteristic of the SOS response. 2-AP does not normally activate the SOS response but, in the absence of ClpXP, it could induce a chain of events that leads to DNA damage. The relevance of a RecA-dependent repair pathway is supported by the observation that *recA clpX* double mutants (NK323) are supersensitive to 2-AP and do not survive low concentrations (40 μg/ml) of 2-AP in the medium. In contrast, a *recA clp*$^+$ *hsd*$^+$ strain (NK308) is no more sensitive to 2-AP than its *rec*$^+$ counterpart (NK301); *recA* strains resemble *rec*$^+$ in their RA response to 2-AP.

Is ClpXP needed in the presence of 2-AP to prevent DNA damage by the resident restriction endonuclease? We made the *clpX* bacteria deficient in restriction both by deleting the *hsd* genes (NK312) and by including a mutation in *hsdR* (NK325), the gene essential for restriction. The restriction-deficient bacteria were not sensitive to 2-AP. Similarly, the hypersensitivity of the *recA clpX* strain was relieved by inactivation of the endonuclease activity. We suggest that during prolonged treatment with 2-AP, the ClpXP-dependent pathway is essential to prevent *Eco*KI from causing DNA damage and consequent cell death.

RA Induced by 2-AP Is Associated with a Deficiency of HsdR. RA is not correlated with a loss of modification activity (14, 30). It could, therefore, be the result of a deficiency in HsdR and the consequent depletion of *Eco*KI ($R_2M_2S_1$), but not the modification enzyme (M_2S_1).

The HsdR and HsdM subunits were monitored by Western blots after the addition of 2-AP to both *clp*$^+$ and *clpX* bacteria (Fig. 3). After a lag of 20 min, a reduction in the concentration of HsdR, but not HsdM, was detected. This deficiency of HsdR was found only in *clp*$^+$ cells in response to 2-AP. RA, therefore, correlated with a ClpX-dependent reduction in the concentration of HsdR, the polypeptide essential for restriction, but not modification.

HsdR Is Degraded in *clp*$^+$ Cells Treated with 2-AP. The very low concentration of HsdR detected in Clp$^+$ cells after a period of growth in the presence of 2-AP (Fig. 3) is consistent with the degradation of HsdR in the presence of ClpXP, but it could be argued that ClpXP in some way affects the synthesis rather than the degradation of HsdR.

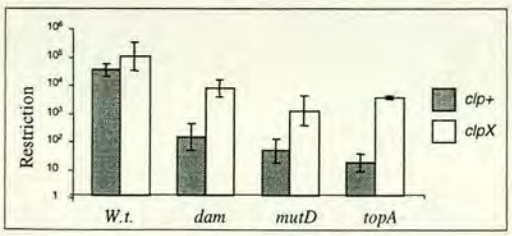

FIG. 2. Restriction of unmodified phage λ by *dam* (NK302), *mutD* (NK326), and *topA* (RS2) strains and their *clpX* derivatives (NK315, NK327, and NK329). It is known that *topA* strains accumulate compensatory mutations in *gyrA* or *gyrB* (21), but the *topA10* strain (RS2) is not known to have a compensatory mutation (21), and the *topA* mutation itself correlates with impaired restriction (G. P. Davies, personal communication).

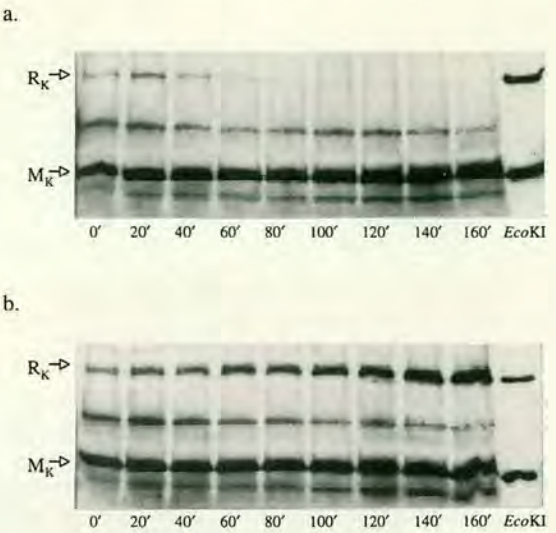

FIG. 3. Assays for HsdR and HsdM polypeptides after treatment with 2-AP. (*a*) *clp*$^+$ bacteria (NK301). (*b*) *clpX* bacteria (NK304). In the absence of 2-AP (data not shown), the assays for *clp*$^+$ and *clpX* bacteria were indistinguishable from those seen in *b*. *Eco*KI polyclonal antibody, used in these Western blots, fails to detect HsdS, but detects HsdR and HsdM and some other *E. coli* proteins.

We therefore assayed the stability of HsdR in *clp*$^+$ and *clpX* cells in response to treatment with 2-AP. The preferred experiment was to rely on the chromosomal *hsdR* gene, but the signal generated from a single copy of *hsdR* was weak compared with those generated by other proteins. Gene dosage was increased by cloning *hsdR* in pACYC184, a low-copy-number vector. *clp*$^+$ *hsd*$^+$ (NK301) and *clpX hsd*$^+$ (NK304) bacteria transformed with the *hsdR*$^+$ plasmid (pNK3) were treated with 2-AP for 90 min to allow the establishment of RA before they were pulse-labeled with [^{35}S]methionine. HsdR was unstable in Clp$^+$ but not ClpX$^-$ cells after 2-AP treatment (Fig. 4). In the absence of 2-AP (data not shown) the HsdR polypeptide was stable in *clp*$^+$ and *clpX* cells for at least 180 min.

These results are consistent with 2-AP as the activator of a RA pathway in which HsdR is susceptible to ClpXP-dependent proteolysis.

Functional *Eco*KI Is Obligatory for the Loss of HsdR That Is Characteristic of RA. Is active *Eco*KI necessary to generate the signal that leads to ClpXP-dependent degradation of HsdR? To answer this question we tested whether 2-AP-induced depletion of HsdR occurs in restriction-deficient mutants. One of the mutants tested has a missense mutation in *hsdR* (NK351), and the other (NK352) has a wild-type *hsdR* gene, but *hsdM* and *hsdS* are deleted so that HsdR cannot form an *Eco*KI complex.

HsdR was not depleted in either mutant in response to 2-AP (Fig. 5*a*). This finding implies that a functional endonuclease is required for induction of the pathway that leads to degradation of HsdR. If the products of restriction by a type I enzyme are the stimulus for RA, the endonuclease activity of one R-M system should induce RA for a different system. We tested whether a functional type IB system (*Eco*AI), for which RA is regulated in a ClpXP-dependent manner, induced degradation of the HsdR polypeptide of the inactive type IA system, *Eco*KI.

We transferred F′$hsd_AR^+M^+S^+$ (F′101–301) to the three strains used in the previous experiment (Fig. 5*a*): $hsd_KR^+M^+S^+$, $hsd_KR^-M^+S^+$, and $hsd_KR^+\Delta(MS)$. The transconjugants, both untreated and treated with 2-AP, were

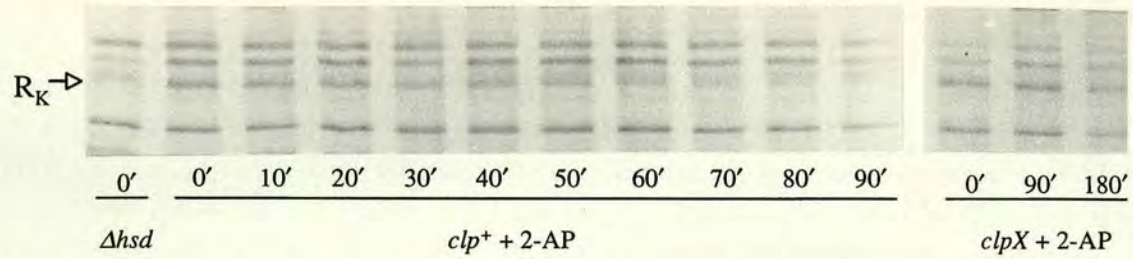

FIG. 4. The stability of HsdR *in vivo* after treatment with 2-AP. Labeled polypeptides separated by electrophoresis through SDS-polyacrylamide gels (6%) were detected by autoradiography. An extract from a strain lacking HsdR (NK311/pACYC184) was analyzed in the first track. Samples from *clp*$^+$ and *clpX* bacteria containing pNK3 were taken at the time intervals indicated after pulse labeling.

assayed for *Eco*AI- and *Eco*KI-dependent restriction *in vivo* and for the presence of HsdR polypeptides. 2-AP caused RA of functional R-M systems, and HsdR from any restriction-proficient complex was lost (Fig. 5*b*). However, for the non-functional *Eco*KI complex, R_K remains even in the presence of functional *Eco*AI. These data require that the stimulus for RA is family-specific and therefore is not simply the product of restriction.

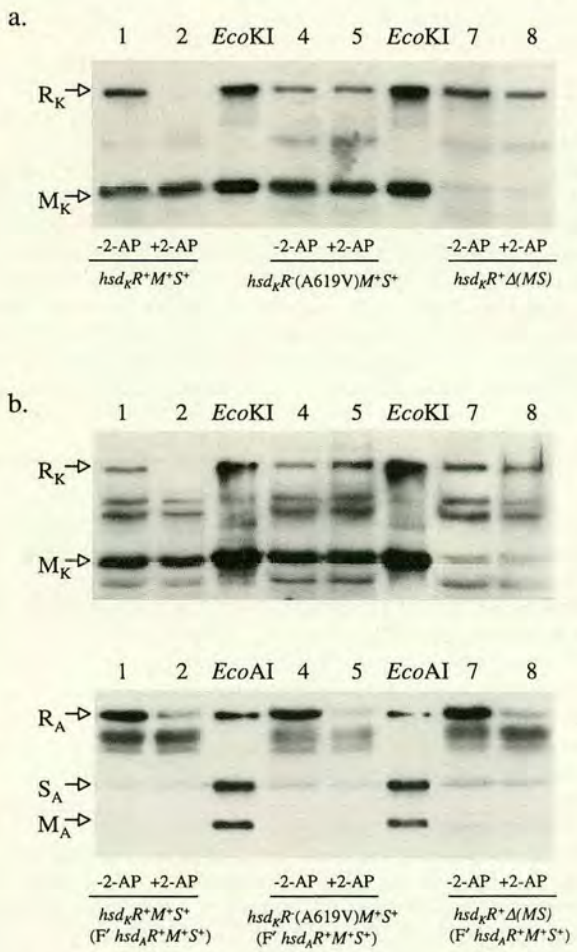

FIG. 5. Hsd subunits were monitored, after treatment with 2-AP, using antibodies raised against the relevant R-M complex. HsdR is degraded only when it is a part of a functional complex. (*a*) Degradation of HsdR is prevented by a missense mutation in *hsdR* (track 5) or by the absence of HsdM and S (track 8). (*b*) The presence of functional *Eco*AI has no effect on the degradation of the HsdR subunit of *Eco*KI (*Upper*), even though the HsdR subunit of *Eco*AI itself is degraded (*Lower*, lanes 2, 5, and 8). The control tracks for *Eco*AI contain a mixture of polypeptides in which HsdM and HsdS are present in molar excess to give strong signals with antibody.

Mutations Predicted to Confer a Restriction-Proficient, Modification-Deficient (r^+m^-) Phenotype Cause Restriction Alleviation. It is logical to expect that a mutation conferring an r^+m^- phenotype would be lethal. We chose to investigate a mutation in *hsdM* (F269G) that abolishes methyltransferase activity but has no effect on the binding of the cofactor *S*-adenosylmethionine and therefore is predicted to leave a functional endonuclease (31). This *hsdM* mutation was transferred from a λ*hsd* phage (λNM1367) to the chromosome of an *hsdR* strain. The presence of *hsdM*(F269G) (NK378) was associated with the anticipated m^- phenotype. We tested the naive prediction that the acquisition of an F′ with a functional *hsdR* gene would generate r^+m^- transconjugants and these would die. However, we found no difference between the survival of the recipients upon acquisition of F′*hsdR*$^+$ and the survival of recipients receiving the control F′ lacking an *hsdR*$^+$

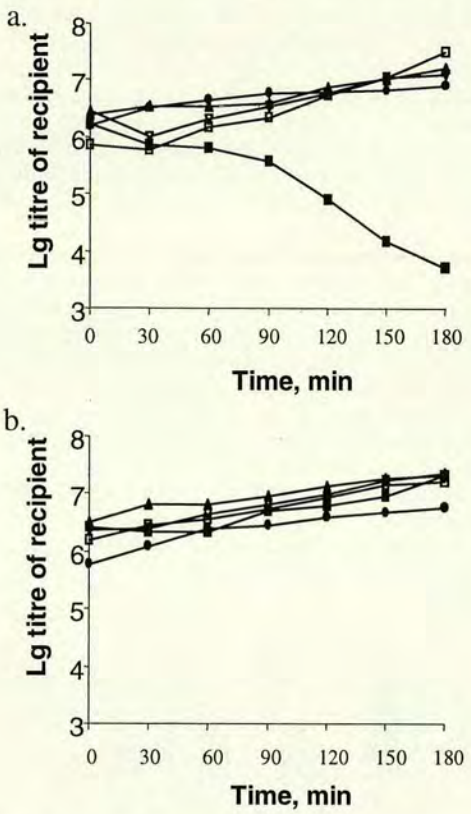

FIG. 6. The survival of *hsdR*$^+$*M*$^-$(F269G)*S*$^+$ cells was assessed after the conjugative transfer of *hsdR*$^+$ to *hsdR*$^-$*M*$^-$*S*$^+$ recipients. (*a*) The experiment using F′101–103 (*hsdR*$^+$). (*b*) The control experiment with F′101–102 (*hsdM*$^+$*S*$^+$). Data are plotted for the following recipients: NK379, *hsdR* (▲); NK380, *hsdR clpX* (▽); NK382, *hsdRM* (□); NK384, *hsdRM clpX* (■); and NK383, *hsdRM recA* (●). The data show that *hsdR*$^+$*M*$^-$(F269G)*S*$^+$ cells survive only if the recipient is ClpX$^+$.

allele (Fig. 6). We extended our experiment to include a *recA* recipient in which DSBs would not be repaired; transfer of the $hsdR^+$ allele still occurred efficiently (Fig. 6).

The EOP of λ.0 on the $hsdR^-M^-S^+$ ($F'hsdR^+$) transconjugants was 10^{-1} in contrast to 5×10^{-4} when the $F'hsdR^+$ was transferred to an $hsdR^-M^+S^+$ recipient (the EOP of λ.K was 1 in both cases). The low level of restriction by the m^- transconjugants is consistent with induction of the RA response. Therefore, the conjugation experiments were extended to include *clpX* recipients. In the absence of ClpX, transfer of the $F'hsdR^+$ to the $hsdR^-M^-S^+$ recipient was lethal, consistent with the presence of functional restriction endonuclease (Fig. 6). Our hypothesis predicts that the transconjugant bacteria can survive in the presence of ClpXP because of the activation of the RA pathway. If this suggestion is correct, $hsdR^+M^-S^+$ bacteria would be deficient in HsdR.

We chose to use chromosomal genes in preference to a plasmid-borne *hsdR* to test this prediction. We transferred the *hsdM*(F269G) mutation to the chromosome of NK301, an hsd^+ strain. The *hsdM* recombinants were recognized by their m^- phenotype and could not be transduced to give *clpX* derivatives (data not shown). These derivatives restricted λ.0 with an efficiency indicative of RA (EOP = 10^{-1}). Consistent with the induction of RA, HsdR was missing in the *hsdM*(F269G) strain that encodes a functional restriction enzyme and present in a derivative with a missense mutation in *hsdR* (Fig. 7). Importantly, when *hsdM*(F269G) was replaced with the wild-type allele (see legend to Fig. 7), HsdR was restored. Therefore, the loss of HsdR is a consequence of the *hsdM* mutation. Our experiments with the modification-deficient mutant show that *E. coli* has an extraordinary capacity to protect itself against potential DNA damage elicited by a resident type I R-M system.

DISCUSSION

The diagnostic feature of RA is an r^- phenotype despite a restriction-proficient genotype (hsd^+). The r^- phenotype that persists for many generations in a transconjugant after the acquisition of functional *hsd* genes by an hsd^- recipient (11, 32) may be viewed as an example of RA. In this case, the establishment of hsd^+ genes in a naive bacterium depends on the ClpXP protease (10). We now have shown that RA in response to a variety of stimuli, including external agents and mutations that affect the fidelity of DNA replication, also requires ClpXP. In two quite different situations the presence of subunits of *Eco*KI was monitored after the induction of RA. In the first, the bacteria were treated with 2-AP, and in the second, a mutation in *hsdM* (F269G) was introduced that blocks only the methyltransferase activity of *Eco*KI (31). In both these examples of ClpXP-dependent RA, a negligible level of HsdR remained. We propose a general pathway for RA in which ClpXP is necessary for the degradation of HsdR and the consequent r^- phenotype. According to this scheme, unmodified chromosomal DNA targets would be a signal for the cell to protect its own DNA from restriction. We believe that all the stimuli for RA examined by us rely on the presence of unmodified target sequences.

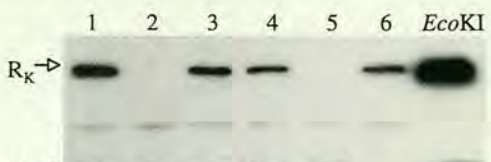

FIG. 7. The effect of *hsdM* (F269G) on the level of HsdR. The mutation *hsdM* (F269G) destroys only the modification activity of *Eco*KI. The level of HsdR was monitored by Western blots by using antibody against *Eco*KI. Lanes 1–6 include extracts of strains. Lanes: 1, NK301 (hsd^+); 2, NK386 [an *hsdM* (F269G) derivative of NK301]; 3, an hsd^+ derivative of NK386; 4, an *hsdR*(A619V) derivative of NK386 (NK388) in which alleles of *hsd* genes were replaced by using λ*hsd* phages that included only *hsdMS* or *hsdR*, respectively; 5, NM802 (an *hsdR* deletion strain); and 6, NK352 (an *hsdMS* deletion strain).

A particularly severe stimulus is provided by the mutation in *hsdM* (F269G) that results in a modification-deficient, restriction-proficient *Eco*KI complex (Fig. 6*a*). For this mutant to survive, despite an unmodified chromosome, restriction alleviation must be extraordinarily effective. A more common stimulus is DNA damage that elicits RecA-dependent repair. UV irradiation and mutations in *dam* can cause DSBs (26, 33); nalidixic acid and mutations in *topA* are likely to generate DSBs by stalling replication. Damage by UV light also leads to lesions in one strand that are repaired postreplicatively (34). RecA-dependent repair relies on homologous recombination. If homologous recombination involves two segments of hemimethylated DNA, the annealing of unmethylated strands or DNA synthesis may generate a localized region of unmethylated DNA. In contrast, both 2-AP and *mutD* increase the frequency of base pair transitions (29, 35). Some mutations will generate new target sequences, all of which will be unmodified.

Our experiments have shown a ClpXP-dependent loss of HsdR in response to 2-AP. It seems likely that the ClpXP protease itself degrades HsdR, rather than being necessary to maintain or activate another protease. The only protease-deficient mutants found to affect the transmission of the genes encoding *Eco*KI were *clpX* and *clpP* (10). Our experiments also show that HsdR is lost only in cells in which HsdR could produce functional *Eco*KI. Thus, in the absence of HsdM and HsdS, wild-type HsdR is not degraded; likewise, in the presence of HsdM and HsdS, a missense mutation in *hsdR* prevents degradation of the nonfunctional polypeptide. The requirement for unmodified targets and functional *Eco*KI might suggest that DNA breakage initiates the RA response. We argue that DSBs are not involved in the initiation of RA. One reason for doubting this idea is our observation that a $recAclp^+hsd^+$ bacterium is no more sensitive to 2-AP than its rec^+ counterpart. This finding is not consistent with the creation of DSBs in response to 2-AP. Second, we tested whether active *Eco*AI, a member of the type IB family of enzymes is sufficient to induce loss of the HsdR subunit of *Eco*KI in response to treatment with 2-AP. It is not, although it is susceptible to ClpXP-dependent RA. If DSBs are the signal for RA, those made by *Eco*AI do not provide a signal for degradation of the HsdR subunit of *Eco*KI. Finally, even in the absence of RecA we readily made strains in which *Eco*KI is defective in methyltransferase activity (Fig. 6). Because DSBs cannot be repaired in a $recA^-$ strain (36), it would appear that in this $hsdR^+M$(F269G) S^+ bacterium DSBs are avoided, despite the presence of ≈600 unmodified targets and the coding potential for restriction-proficient, modification-deficient *Eco*KI. We conclude that ClpXP-dependent degradation of HsdR is able to prevent cutting of the bacterial chromosome. In the absence of ClpXP, however, even rec^+ cells fail to survive because *Eco*KI cuts their chromosomes.

If DSBs are not the stimulus for RA, why does a missense mutation in *hsdR* prevent degradation of HsdR? The amino acid substitution (A619V) is associated with a defect in the hydrolysis of ATP and probably, therefore, with the ATP-dependent translocation of DNA that precedes the generation of DSBs (18). The missense mutation does not prevent either the binding of *Eco*KI to its target sequence or the associated ATP-dependent conformational change that is a prerequisite for the restriction pathway (18, 37). Other missense mutations in HsdR also prevent degradation of HsdR (V.A.D. and N.E.M., unpublished observations); therefore, it seems probable that the functional defect, rather than the amino acid substitution *per se*, determines whether the enzyme is a substrate for ClpXP. We conclude that HsdR is recognized only after the *Eco*KI complex has embarked on its restriction pathway. It remains to be determined what renders the HsdR

subunits susceptible to proteolysis. Nevertheless, the present experiments promote the concept of a remarkably specific control mechanism, effective only once the relevant restriction pathway has been initiated, but able to act before any damage is inflicted on unmodified chromosomal DNA.

The RA response can protect the bacterial chromosome from restriction in the complete absence of modification, but the alleviation is not entirely complete when analyzed by infection with λ.0 (EOP = 10^{-1}). These facts raise two new, but probably related, problems. First, why does phage DNA entering the cell show some susceptibility to restriction whereas the resident bacterial chromosome does not? Second, why do unmodified targets on the chromosome, but not those on incoming phage DNA, stimulate the RA response? At present, it should be borne in mind that the two substrates differ in their location and their association with other proteins.

Our current experiments document the disappearance of HsdR under conditions of RA, and we interpret this as ClpXP-dependent degradation of HsdR. Initial but unsuccessful attempts to detect degradation *in vitro* used purified HsdR, or *Eco*KI, as substrate. The *in vivo* experiments indicate that the substrate is unlikely to be protein alone but, rather, a functional protein–DNA complex.

The role of ClpXP in the disassembly and degradation of the Mu transposase already is known to be complex. MuB apparently protects the MuA–DNA complex from recognition by ClpX and, hence, from disassembly and potential degradation by the protease activity of ClpP (38). These authors suggested "that a protein–complex architecture that uses overlapping sequences for subunit interactions and for targeting a protein for remodeling or destruction provides a useful design for this type of regulation." By analogy we would suggest that some step in the ATP-dependent DNA translocation by *Eco*KI leads to the exposure of the target sequence for ClpX.

Our investigation of the relevance of ClpXP to RA has been confined to the type IA and IB families of R-M systems. There is evidence for Dam-mediated RA of a type III system (26). Members of the type IC and ID families are susceptible to RA in response to 2-AP (unpublished results), but transmission of the plasmid-borne type IC *hsd* genes by conjugation is not dependent on ClpXP (10, 32). Although the assembly pathway of the $R_2M_2S_1$ complex may provide a lag in the production of the endonuclease after plasmid transfer (39), it would not prevent the cutting of unmodified targets created in cells in which functional endonuclease is already assembled. It is not known whether RA can involve other proteases or other mechanisms, but RA is found for some methylation-dependent restriction systems (24), where DNA damage would not generate target sequences. RA has not been detected for any type II system; rather, RA appears to be characteristic of complex R-M systems.

Our experiments demonstrate that control of the restriction activity of *Eco*KI is extraordinarily sensitive. It not only copes with the acquisition of *hsd* genes conferring new specificities and the production of unmodified targets created by repair and mutation, but *clp*$^+$ cells also survive a mutation that destroys the modification activity of the R-M complex. A similar control system could permit the efficient phase variation of type I R-M systems, a phenomenon recently documented for *Mycoplasma pulmonis* (40). Molecular mechanisms of the sophisticated interactions that mediate the proteolytic control remain to be determined.

We thank our colleagues for their interest throughout this work and, most particularly, Susan Gottesman, David R. F. Leach, Mary O'Neill, Lynn M. Powell, and Frank W. Stahl for their constructive criticism of the manuscript. We are indebted to David Leach for strains and Pavel Janscak for *Eco*AI. S.M. and V.A.D. thank the Darwin Trust for the award of Scholarships. The research was supported by the Medical Research Council.

1. Barcus, V. A., Titheradge, A. J. & Murray N. E. (1995) *Genetics* **140,** 1187–1197.
2. Redaschi, N. & Bickle, T. A. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology*, eds. Neidhardt, F. C., Curtiss, R., Ingraham, J. L., Lin, E. C. C., Low K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. & Umbarger, H. E. (Am. Soc. Microbiol., Washington, DC), pp. 773–781.
3. Murray, N. E., Gough, J. A., Suri, B. & Bickle, T. A. (1982) *EMBO J.* **1,** 535–539.
4. Price, C., Pripfl, T. & Bickle, T. A. (1987) *Eur. J. Biochem.* **167,** 111–115.
5. Titheradge, A. J., Ternent, D. & Murray, N. E. (1996) *Mol. Microbiol.* **22,** 437–447.
6. Boyer, H. W. & Roulland-Dussoix, D. (1969) *J. Mol. Biol.* **41,** 459–472.
7. Fuller-Pace, F. V., Cowan, G. M. & Murray, N. E. (1985) *J. Mol. Biol.* **186,** 65–75.
8. Prakash-Cheng, A., Chung, S. S. & Ryu, J. (1993) *Mol. Gen. Genet.* **241,** 491–496.
9. O'Neill, M., Chen, A. & Murray, N. E. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 14596–14601.
10. Makovets, S., Titheradge, A. J. & Murray, N. E. (1998) *Mol. Microbiol.* **28,** 25–35.
11. Prakash-Cheng, A. & Ryu, J. (1993) *J. Bacteriol.* **175,** 4905–4906.
12. Bertani, G. & Weigle, J. J. (1953) *J. Bacteriol.* **65,** 113–121.
13. Thoms, B. & Wackernagel, W. (1984) *Mol. Gen. Genet.* **197,** 297–303.
14. Efimova, E. P., Delver, E. P. & Belogurov, A. A. (1988) *Mol. Gen. Genet.* **214,** 317–320.
15. Diaz, R., Barnsley, P. & Pritchard, R. H. (1979) *Mol. Gen. Genet.* **175,** 151–157.
16. King, G. & Murray, N. E. (1995) *Mol. Microbiol.* **16,** 769–777.
17. Webb, J. L., King, G., Ternent, D., Titheradge, A. J. & Murray, N. E. (1996) *EMBO J.* **15,** 2003–2009.
18. Davies, G. P., Powell, L. M., Webb, J. L., Cooper, L. P. & Murray, N. E. (1998) *Nucleic Acids Res.* **26,** 4828–4836.
19. Maurizi, M. R., Clark, W. P., Katayama, Y., Rudikoff, S., Pumphrey, J., Bowers, B. & Gottesman, S. (1990) *J. Biol. Chem.* **265,** 12536–12545.
20. Gottesman, S., Clark, W. P., de Crecy-Lagard, V. & Maurizi, M. R. (1993) *J. Biol. Chem.* **268,** 22618–22626.
21. DiNardo, S., Voelkel, K. A., Sternglanz, R., Reynolds, A. E. & Wright, A. (1982) *Cell* **31,** 43–51.
22. Sain, B. & Murray, N. E. (1980) *Mol. Gen. Genet.* **180,** 35–46.
23. Chang, A. C. Y. & Cohen, S. N. (1978) *J. Bacteriol.* **134,** 1141–1156.
24. Kelleher, J. E. & Raleigh, E. A. (1994) *J. Bacteriol.* **176,** 5888–5896.
25. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
26. Efimova, E. P., Delver, E. P. & Belogurov, A. A. (1988) *Mol. Gen. Genet.* **214,** 313–316.
27. Wang, T. C. & Smith, K. C. (1986) *J. Bacteriol.* **165,** 1023–1025.
28. Michel, B., Ehrlich, S. D. & Uzest, M. (1997) *EMBO J.* **16,** 430–438.
29. Echols, H., Lu, C. & Burgers, P. M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2189–2192.
30. Hiom, K. J. & Sedgwick, S. G. (1992) *Mol. Gen. Genet.* **231,** 265–275.
31. Willcock, D. F., Dryden, D. T. & Murray, N. E. (1994) *EMBO J.* **13,** 3902–3908.
32. Kulik, E. M. & Bickle, T. A. (1996) *J. Mol. Biol.* **264,** 891–906.
33. Bonura, T. & Smith, K. C. (1975) *J. Bacteriol.* **121,** 511–517.
34. Rupp, W. D. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology*, eds. Neidhardt, F. C., Curtiss, R., Ingraham, J. L., Lin, E. C. C., Low K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. & Umbarger, H. E. (Am. Soc. Microbiol., Washington, DC), pp. 2277–2294.
35. Ronen, A. (1980) *Mutat. Res.* **75,** 1–47.
36. Krasin, F. & Hutchinson, F. (1977) *J. Mol. Biol.* **116,** 81–98.
37. Powell, L. M., Dryden, D. T. & Murray, N. E. (1998) *J. Mol. Biol.* **283,** 963–976.
38. Levchenko, I., Yamauchi, M. & Baker, T. A. (1997) *Genes Dev.* **11,** 1561–1572.
39. Janscak P., Dryden, D. T. & Firman, K. (1998) *Nucleic Acids Res.* **26,** 4439–4445.
40. Dybvig, K., Sitaraman, R. & French, C. T. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 13923–13928.